Isolation of salt-tolerant, iron-oxidising, acidophilic bacteria and assessment of their bioleaching potential at high salinity

Patricia E. Linton B.Sc.(Hons)

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School of Life Sciences
Heriot-Watt University
Edinburgh

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Abstract

The occurrence of chemoautotrophic, acidophilic bacteria in the marine environment has been widely noted and they have been implicated in the biogeochemical cycling of iron and biodeterioration of iron-containing structures in the oceans. However, the isolation, molecular ecology, growth profiles and physiological responses of these bacteria at elevated salt levels have rarely been described, despite widespread interest in their unique metabolic capacity. These bacteria may have a potential application in the extraction of metals via bioleaching of salt contaminated ores or to facilitate the use of seawater in the bioleaching process. Traditional bioleaching microorganisms cannot be used in these cases due to the toxicity of elevated salt concentrations. In this study, three strains of halotolerant gram-positive, rod shaped, acidophilic bacteria were isolated from estuarine and coastal areas, two of which were novel species. Enrichment cultures were set up using pyrite medium of different salinities with sediment and seawater samples from a variety of metal contaminated areas exposed to the sea or brackish water. These enrichment cultures were then further purified using end-point dilution culture methods and the 16S rRNA genes were sequenced and phylogeny assessed. The growth characteristics, morphology and growth profiles on a variety of metaliferrous ore samples of the strains were characterised. The strains exhibited autotrophic growth on a variety of iron and sulphur-containing compounds, heterotrophic growth on yeast extract medium as well as mixotrophic growth on a combination of these substrates. The strains grew optimally with $30gl^{-1}$ sea salts added to the medium, at a pH of 2.0 and a temperature of 37°C. Two of these isolated bacteria represent novel species in the genera *Sulfobacillus* and *Alicyclobacillus*. High final iron dissolution levels were demonstrated after biooxidation of Lihir gold ore and Escondida Copper ore in medium with 30 gl^{-1} sea salts and 2% ore for 30 days. Bacterium 4G mediated 66.10%, 5C 100% and Cligga 88.86% dissolution of the total iron present in the Lihir sample after 30 days, while bacterium 4G mediated 52.63%, 5C 60% and Cligga 49.75% dissolution of the total iron present in the Escondida samples after 30 days. The growth characteristics displayed by these bacterial strains demonstrate their potential application in high salinity bioleaching operations.

'Research is the process of going up alleys to see if they are blind'

(Marston Bates, Zoologist. 1906-1974)

For Irene and Joe

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'Turn your face to the sun and the shadows fall behind you'

(Maori proverb)

Table of Contents

Section	Title	Page
	Abstract	i
	Dedication	iii
	Acknowledgements	iv
	Table of Contents	v
	Glossary	xiii
1	**Chapter One: Introduction**	**1**
1.1	**Diversity of Acidophilic prokaryotes**	**2**
1.1.1	*The Genus Acidithiobacillus*	4
1.1.2	*The Genus Leptospirillum*	4
1.1.3	*The Genus Sulfobacillus*	5
1.1.4	*The Genus Alicyclobacillus*	6
1.1.5	*The Genus Sulfolobus*	6
1.1.6	*The diversity of acidophiles in acid mine drainage and their role in its formation*	7
1.2	**Chemoautotrophy in acidophilic bacteria**	**8**
1.2.1	*Metabolism of pyrite/iron-disulphide (FeS_2)*	9
1.2.2	*Metabolism of ferrous iron*	11
1.3	**The case for the widespread existence of halotolerant acidophilic bacteria**	**13**
1.3.1	*Diversity of sulphur-oxidising bacteria from high salt environments*	13
1.3.2	*Diversity of iron-oxidising bacteria from high salt environments*	14
1.4	**Biomining: theory and industrial practices**	**22**
1.4.1	*Mechanism of bacterially mediated metal extraction*	22
1.4.2	*Types of biomining operations*	26
1.5	**The case for biomining at high salinity**	**29**
1.5.1	*Bioleaching and biooxidation attempts at high salinity*	30
1.7	**Aims and Objectives of the Study**	**33**
2	**Chapter Two - Methods and Materials**	**35**
2.1	**Characterised bacterial strains used in this study**	**35**
2.2	**Media and culture conditions**	**37**
2.2.1	*T. prosperus medium*	37

2.2.2	*At. ferrooxidans medium*	37
2.2.3	*Media used for the enrichment and growth of isolated bacteria*	37
2.2.4	*Ferrous iron Overlay Plates*	38
2.2.5	*Solid ferrous iron medium*	39
2.2.6	*Estuarine Enrichment medium (EEM)*	39
2.2.7	*Alicyclobacillus hesperdum medium (DSMZ 12489)*	40
2.2.8	*Alicyclobacillus cycloheptanicus medium (DSMZ 4006)*	40
2.2.9	*Sulfobacillus acidophilus medium (DSMZ 10332)*	40
2.2.10	*Sulfobacillus disulfidooxidans medium (DSMZ 12064)*	40
2.2.11	*Acidophilium spp. medium (NCIMB 11745)*	41
2.2.12	*Leptospirillum ferrooxidans medium (ATCC 53992)*	41
2.2.13	*TSM solid medium*	41
2.3	**Monitoring of cell growth**	**42**
2.3.1	*Direct counting of planktonic cells*	42
2.3.2	*Specific growth rate constant calculation*	42
2.3.3	*Use of fluorescence stain to count cells under UV*	43
2.3.4	*Use of oxalic acid for release of cells attached to particles or which are enclosed in iron precipitates*	43
2.3.5	*Use of sonic water bath to release cells attached to ore particles*	43
2.3.6	*Measurement of growth in heterotrophic bacterial cultures*	44
2.4	**Determination of ferrous iron and total dissolved iron concentrations in bacterial cultures**	**45**
2.4.1	*Ferrous iron determination using 1,10-phenanthroline indicator*	45
2.4.2	*Total iron determination using 1,10-phenanthroline indicator*	46
2.4.3	*Preparation of standard curves for use with 1,10-phenanthroline indicator iron determination method*	46
2.4.4	*Solutions used in iron determination*	47
2.5	**DNA extraction from bacterial ore cultures**	**48**
2.5.1	*Preparation of bacterial pellets from ore cultures*	48
2.5.2	*Ethanol/sodium acetate precipitation of DNA*	48
2.5.3	*PCR reagents and conditions*	49
2.6	**Preparation of PCR products for DNA sequencing**	**51**
2.6.1	*Purification of PCR products for sequencing*	51
2.6.2	*Cycle sequencing reagents*	51
2.6.3	*Cycle sequencing reaction conditions*	52

2.6.4	*Purification of sequencing reaction*	52
2.6.5	*Denaturation of sequencing reactions*	52
2.6.6	*Sequence determination*	53
2.7	**Phylogenetic Characterisation of Environmental Isolates**	**54**
2.7.1	*Determination of the sequence of 16S rDNA*	54
2.7.2	Assembly of contiguous 16S rRNA DNA sequences	54
2.8	**Environmental Sampling to isolate salt tolerant iron-oxidising bacteria**	**56**
2.8.1	*Sampling in East Fife, Scotland, UK*	56
2.8.2	*Sampling in Cornwall, England, UK*	56
2.8.3	*Sample processing*	59
2.8.4	*Enrichment of bacteria from environmental samples*	60
2.8.5	*Solid isolation media*	60
2.9	**Morphology and growth physiology of environmental isolates**	**62**
2.9.1	*Gram stain*	62
2.9.2	*Light microscopy*	62
2.9.3	*Electron microscopy*	63
2.9.4	*Sporulation*	63
2.9.5	*Growth physiology*	63
2.10	**Composition of Rio Tinto ore samples**	**65**
2.10.1	*Spanish chalcopyrite*	65
2.10.2	*Lihir gold ore*	66
2.10.3	*Freeport rough feed copper ore*	67
2.10.4	*Freeport final concentrate*	68
2.10.5	*Somincor copper ore*	69
2.10.6	*Escondida copper ore*	70
2.11	**Assessment of the biooxidation potential of the environmental isolates**	**71**
2.11.1	*Medium and culture conditions*	71
2.11.2	*Measurements taken during biooxidation experiments*	71

3	**Chapter Three: Isolation and characterisation of iron-oxidising bacteria that are both acidophilic and halotolerant**	**74**
3.1	**Introduction**	**74**
3.2	**Enrichment of iron-oxidising, acidophilic, halotolerant bacteria**	**76**
3.2.1	*Introduction*	76
3.2.2	*Liquid enrichment and isolation media*	78
3.2.3	*Solid enrichment and isolation media*	79
3.3	**Morphology of isolated bacteria**	**84**
3.3.1	*Introduction*	84
3.3.2	*Morphology*	84
3.4	**Growth of the isolated bacteria at different concentrations of sea salts**	**88**
3.4.1	*Introduction*	88
3.4.2	*Growth of isolate 4G at different levels of salinity*	89
3.4.3	*Growth of isolate 5C at different levels of salinity*	89
3.4.4	*Growth of isolate Cligga at different levels of salinity*	90
3.4.5	*Exponential growth rate constants of 4G, 5C, Cligga on different salt sources*	90
3.5	**Growth of the isolated bacteria under mixotrophic and heterotrophic conditions**	**96**
3.5.1	*Introduction*	96
3.5.2	*Growth of isolate 4G under mixotrophic and heterotrophic conditions*	96
3.5.3	*Growth of isolate 5C under mixotrophic and heterotrophic conditions*	97
3.5.4	*Growth of isolate Cligga under mixotrophic and heterotrophic conditions*	97
3.5.5	*Specific growth rate constants of isolates 4G, 5C and Cligga*	97
3.6	**Optimum temperature for growth of the isolated bacteria**	**102**
3.6.1	*Introduction*	102
3.6.2	*Growth rate constants of 4G, 5C and Cligga at different temperatures*	102

3.7	**Optimum pH for growth of isolated bacteria**	**104**
3.7.1	*Introduction*	104
3.7.2	*Growth rate constants of 4G, 5C and Cligga at different initial pH*	104
3.8	**Growth of the isolated bacteria on solid medium**	**106**
3.8.1	*Introduction*	106
3.8.2	*Growth of environmental isolates on solid medium*	107
3.9	**Growth of the isolated bacteria on ferrous iron, tetrathionate and elemental sulphur**	**112**
3.9.1	*Introduction*	112
3.9.2	*Growth of the bacteria on different substrates*	112
3.10	**Assessment of *T. prosperus* for use as a comparative benchmark microorganism for subsequent growth experiments with the isolated salt-tolerant bacteria**	**114**
3.10.1	*Introduction*	114
3.10.2	Assessment of enumeration methods for T. prosperus	114
3.10.3	*Maintenance of T. prosperus*	115
3.10.4	*Growth of T. prosperus and At, ferrooxidans at high salinity*	116
3.11	**Discussion**	**118**
3.11.1	*Enrichment of target bacteria*	118
3.11.2	*Growth of the bacterial isolates on different concentrations of sea salts*	120
3.11.3	*Comparison of the growth of the bacteria under mixotrophic and heterotrophic conditions*	123
3.11.4	*Effect of temperature on growth of the bacteria*	124
3.11.5	*Effect of initial medium pH on growth of the bacteria*	125
3.11.6	*Growth of the bacterial isolates on solid medium and other substrates*	127
	Chapter Summary	129
4	**Chapter Four: Phylogenetic characterisation of the environmental bacterial isolates**	**131**
4.1	**Introduction**	**131**
4.2	**16S rDNA sequences of the three isolated bacterial strains**	**133**
4.2.1	*Introduction*	133

4.2.2	*Sequences of 16S rRNA gene from isolated bacterial strains, 4G, 5C and Cligga*	134
4.3	**Similarity matrixes of 16S rDNA of isolated bacteria and other closely related bacteria**	**137**
4.3.1	*Introduction*	137
4.3.2	*Sequence similarities obtained for isolated strains 4G, 5C and Cligga*	132
4.4	**Phylogenetic tree construction for the isolated acidophilic bacteria and other related bacteria**	**143**
4.4.1	*Introduction*	143
4.4.2	*Phylogenetic trees showing the relationships of the isolated bacteria with other similar bacteria*	143
4.5	**Discussion**	**153**
5	**Chapter Five: Assessment of bioleaching potential at high salinity**	152
5.1	**Introduction**	**152**
5.2	**Bioleaching of Las Cruces Spanish copper ore by the isolated bacteria**	**159**
5.2.1	*Introduction*	159
5.2.2	*Growth and iron dissolution kinetics of the isolates grown on Las Cruces ore*	159
5.3	**Biooxidation of Lihir gold ore by isolated bacteria**	**166**
5.3.1	*Introduction*	166
5.3.2	*Growth and iron dissolution kinetics of the isolated bacteria when grown on Lihir gold ore*	167
5.4	**Bioleaching of the Freeport Rough Feed ore sample by the isolated bacteria**	**173**
5.4.1	*Introduction*	173
5.4.2	*Growth and iron dissolution kinetics of the isolated bacteria when grown on Freeport Rough Feed ore*	173
5.5	**Bioleaching of Freeport Final concentrate by the isolated bacteria**	**179**
5.5.1	*Introduction*	179

5.5.2	*Growth and iron dissolution kinetics of the isolated bacteria when grown on Freeport Final concentrate ore*	179
5.6	**Bioleaching of Somincor ore sample by the isolated bacteria**	**184**
5.6.1	*Introduction*	184
5.6.2	*Growth and iron dissolution kinetics of the isolated bacteria when grown on Somincor copper ore*	184
5.7	**Bioleaching of Escondida ore sample by the isolated bacteria**	**190**
5.7.1	*Introduction*	190
5.7.2	*Growth and iron dissolution kinetics of the isolated bacteria when grown on Escondida copper ore*	190
5.8	**Comparison of the total iron dissolution from different metaliferrous ores mediated by the isolated bacterial strains**	**196**
5.9	**Discussion**	**197**
6	**Chapter Six: Discussion**	**201**
6.1	**Isolation sites**	**201**
6.2	**Isolation media**	**203**
6.3	**Growth physiology**	**205**
6.4	**Phylogenetics of the isolated bacteria**	**210**
6.5	**The potential use of the isolated bacteria in biomining at elevated salinity**	**211**
6.6	**Suggestions for further study**	**214**
6.7	**Conclusions**	**216**
	References	**218**
	Appendices	**237**
A	**Maps of sampling locations**	**237**
Ai	Location of sampling sites within Cornwall, UK	237
Aii	Map of Calenick Creek sampling location	238
Aiii	Map of Cligga Head sampling location	238
Aiv	Map of Devoran sampling location	239
Av	Map of The Gannel sampling location	239
Avi	Map of Hayle Estuary sampling location	240
Avii	Map of Restronguet Creek sampling location	240
Aviii	Map of St. Michaels's Mount sampling location	241
Aix	Map of Tintagel Head sampling location	241

Ax	Map of Tressillian sampling location	242
Axi	Map of Trevone sampling location	242
B	**Mineralogical reports of Lihir and Escondida ore**	**243**
Bi	Lihir ore	243
Bii	Escondida ore	252
C	**Standard curves for iron determination**	**264**
Ci	Standard curve for the determination of total iron concentration using the pyrite method	264
Cii	Standard curve for the determination of ferrous iron concentrations using the pyrite method	264
D	**Chemicals used in this study**	**265**

Glossary:

Acid Mine Drainage/AMD – water containing sulphuric acid, dissolved metals and ferric iron complexes caused by bacterial activity at mine sites, coal spoil heaps or areas of pyritic mineral geology.

Acidophiles – microorganisms with a pH optimum for growth of under pH 5.0

Arsenopyrite – ore of the chemical composition FeAsS

Biogeochemical cycling – the biologically mediated transformation of elements that results in their global cycling

Biohydrometallurgy - the use of microorganisms in the metals industry

Bioleaching – the conversion of an insoluble metal into a soluble form (e.g. from a metal sulphide into a metal sulphate

Biomining – the use of microorganisms in the extraction of metals from ores

Biooxidation – process in which the recovery of a metal is enhanced by microbial decomposition of the mineral but the target metal is not solubilised and subsequently requires the use of traditional methods for recovery of this metal

Chalcocite – ore of the chemical composition Cu_2S

Chalcopyrite – ore of the chemical composition $CuFeS_2$

Chemolithotrophic/ Chemoautotrophic – organisms that derive energy from the oxidation of inorganic compounds for the assimilation of simple materials and get cellular carbon by the reduction of CO_2

Dump leaching – method of biomining where the ore to be leached is placed on a slope and irrigated with lixivant, the leachate is then collected at the bottom of the dump

Electrowinning - extraction of electrolytic metal from a molten salt or solution

Endospores – thick walled spores formed within a parent cell as a direct response to stresses

Extremophiles – microorganisms characterised by extremes in growth conditions, including temperature, pH, water availability, pressure, salinity

Fumarole – small volcanic vent issuing gases but no lava

Halophiles – microorganisms requiring NaCl for growth

Halotolerant – microorganisms which can tolerate or grow in the presence of elevated salt concentrations

Heap leaching – method of biomining where the ore material to be leached is heaped in layers, these layers are usually aerated and irrigated with lixivant

Heterotrophs – microorganisms that derive energy from the oxidation of organic compounds

Hypersaline/salt lake – an enclosed body of water in areas of inland drainage with a concentration of salts that is much higher than in ordinary river water (sometimes nearing saturation)

Karst environments - terrain with special landforms and drainage characteristics due to greater solubility of certain rocks in natural waters than is common, such as cave environments

Leach liquor/ Lixivant - liquid applied to ore in bioleaching or chemical-leaching processes

Leachate – solution containing soluble target metals after the process of leaching

Leaching – the washing or extraction of soluble constituents from insoluble materials

Mesophiles – microorganisms whose optimum growth is in the temperature range 20°C - 40°C

Metallurgy – the science and technology of metals and their alloys including methods of extraction and use

Mixotrophs – microorganisms capable of utilising both autotrophic and heterotrophic metabolic processes

Ore – metaliferrous mineral from which the metal can be profitably extracted

Psychrophiles – microorganisms whose optimum growth temperature is below 20 °C

Pyrite – ore of the composition FeS_2

Recalcitrant ore – ore which is difficult to treat with traditional chemical and biological methods

Redox potential/oxidation-reduction potential (Eh) – measure of the tendency of a given system to donate electrons (i.e. act as a reducing agent) or to accept electrons (i.e. act as an oxidising agent). The Eh (measured in millivolts/mV) of a given system may be determined by measuring the electrical potential difference between that system and a standard hydrogen electrode (0 volts)

Salt marsh - a marsh characterised by saline soil most often in estuaries and subject to marine inundation

Salt petre – inland natural salt deposits composed of sodium, potassium and nitrate salts

Solfatora – hot, sulphur-rich environment; a volcanic area or vent which yields sulphur vapours and steam

Thermophiles – microorganisms whose optimum growth temperature is above 40°C

Abbreviations used:

AMD – acid mine drainage

DGGE – denaturing gradient gel electrophoresis

FSM –ferrous iron saline medium

ND – not determined

ppm – parts per million

ppt – parts per thousand

PSM – pyrite saline medium

SS – sea salts

YSM – yeast extract saline medium

Chapter One

Introduction

Chapter One: Introduction

Many scientists have offered theories on the origins of life on this planet and the environments in which this early life began. One widely considered view is that present day 'extreme' environments were the norm on primitive earth and that the microorganisms that inhabit such environs today may be some of the oldest and therefore most highly adapted life forms on present day earth. It has been postulated that present day harsh environments such as deep sea hydrothermal vents, hot geysers and fumaroles, subterranean karst environments and environments with high levels of acid mine drainage and metal concentrations, to name but a few, are reminiscent of the conditions encountered on primitive earth. New microorganisms that push the boundaries of our considered views of the limits of life on this planet are continually being discovered. This extraordinary and diverse group of microorganisms has provided a plentiful resource for the biotechnology industry and even influenced man to re-examine the possibilities of microbial life on other planets with similar extreme environments, even spawning a whole new area of science, that of astrobiology.

It is postulated that those microorganisms that survive and thrive in such extreme environments today are able to do so because of millions of years of adaptation, enabling the evolution of physiological processes effective in such environments. This adaptation has enabled these microbes to inhabit niches that, although they appear extreme compared to most branches of life, are anything but for these fascinating groups of extremophilic microorganisms. These extremes include temperature, pH, salinity, osmotic stress, oxygen, low-nutrient environments, and high levels of toxic metals. Interestingly, many extremophiles thrive in environments with more than one extreme.

1.1 Diversity of acidophilic prokaryotes

Acidophilic, chemoautotrophic microorganisms are one such group of extremophiles and these microorganisms are responsible for most of the primary productivity in acidic environments. They are ubiquitous in environs such as acidic springs and geysers, areas of acid mine drainage pollution, sulphidic mineral mine sites and hydrothermal vent areas.

Extreme acidophiles are described as being those microorganisms that have pH growth optima below pH 3.0 (Norris & Johnson, 1998). Despite their harsh external conditions, these acidophiles usually maintain an internal pH of 6.0 – 7.0 via a complex mechanism involving the transmembrane electric potential (Suzuki *et al*, 1999). All cellular life uses chemiosmosis to make energy, which is usually in the form of ATP, this depends on proton gradients, which may be altered by extreme extracellular pH. There is a net force on protons called the proton motive force, which is the sum of the forces from the extra-cytosolic concentration gradient and a charge separation. The cell depends on a net force driving protons into the cell and when such a proton motive force exists, the cell can let protons into the cell through an ATP synthesis protein complex, which uses energy released from the proton movements to create a phosphate bond. A chemolithotrophic bacterial cell keeps this cycle going by using chemical energy gained by the oxidation of inorganic chemicals (see Figure 1.2) to pump protons out of the cell via an electron transport chain (Dilworth & Glenn, 1999). However, acidophilic bacteria have to use more energy to pump sufficient protons out of the cell because of the large proton gradient.

Although the precise structural and biochemical adaptations employed by these microorganisms to thrive in such low pH environments have not been fully elucidated, they have been subject to intense research and review (Alexander *et al*, 1987; Cox *et al*, 1979; Driessen et al, 1996 and Hsung & Haug. 1975).

Acidophilic prokaryotes are widespread in nature and represent a diverse group of microorganisms both phylogenetically and physiologically. The most widely characterised groups include the gram-negative proteobacteria, low G+C gram-positive bacteria, members of the Nitrospira and many examples of the Kingdom Archea. This

section gives an overview of the most widely characterised microorganisms from these groups. Table 1.1 gives an overview of acidophilic bacteria.

Table 1.1 Examples of acidophilic microorganisms (compiled from Hallberg & Johnson, 2001; Johnson, 1998 and Krebs *et al*, 1997)

Microorganism	Phylogenetic affiliation	Mode of nutrition
Mesophiles		
Acidithiobacillus ferrooxidans	β/γ -Proteobacteria	Iron and sulphur-oxidising
Thiobacillus prosperus	γ-Proteobacteria	Iron-oxidising
Leptospirillum ferrooxidans	Nitrospira	Iron-oxidising
Acidithiobacillus thiooxidans	γ-Proteobacteria	Sulphur-oxidising
Thiobacillus albertis	unknown	Sulphur-oxidising
Thiobacillus acidophilus	α-Proteobacteria	Sulphur-oxidising
Sulfobacillus disulfidooxidans	Gram-positive division	Autotrophic, mixotrophic or heterotrophic
Acidiphilium spp.	α-Proteobacteria	Heterotrophic
Acidocella spp.	α-Proteobacteria	Heterotrophic
Acidobacterium capsulatum	unknown	Heterotrophic
Acidmicrobium ferrooxidans	Actinobacteria	Autotrophic, mixotrophic or heterotrophic
Ferroplasma acidiphilum	Thermoplasmales - Archea	Iron-oxidising
Moderate thermophiles		
Sulfobacillus acidophilus	Gram-positive division	Autotrophic, mixotrophic or heterotrophic
Acidithiobacillus caldus	γ-Proteobacteria	Sulphur-oxidising
Sulfobacillus thermosulfidooxidans	Gram-positive division	Autotrophic, mixotrophic or heterotrophic
Acidimicrobium ferrooxidans	Actinobacteria	Iron-oxidising/reducing
Leptospirillum thermoferrooxidans	unknown	Iron-oxidising
Alicyclobacillus spp.	Gram-positive division	Heterotrophic
Thermoplasma spp.	Thermoplasmales - Archea	Heterotrophic
Extreme thermophiles		
Sulfolobus shibitae	Sulfobales - Archea	Mixotrophic
Sulfolobus solfataricus	Sulfobales - Archea	Mixotrophic
Sulfolobus metallicus	Sulfobales - Archea	Iron and sulphur-oxidising
Metallosphaera spp.	Sulfobales - Archea	mixotrophic
Acidianus brierleyi	Sulfobales - Archea	Iron-oxidising

1.1.1 The Genus Acidithiobacillus

This genus has received the most widespread and in-depth study of all the acidophiles, covering their physiology, molecular systems, use in biomining processes and as the catalysts of environmental damage such as acid mine drainage. At the forefront of this research is *Acidithiobacillus ferrooxidans*, which was known as *Thiobacillus ferrooxidans* until recently, when it was reclassified (Kelly & Wood, 2000). This species falls between the β and γ subgroups of the proteobacteria and is a mesophilic, gram-negative, rod-shaped acidophile. *At. ferrooxidans* has a pH optimum between 1.0 and 4.5 (depending on the strain) and a temperature optimum of 30°C. The bacterium derives energy and reducing power from the oxidation of ferrous iron and reduced sulphur compounds and obtains its cellular carbon by fixing atmospheric carbon dioxide (Leduc & Ferroni, 1994). The principle component that facilitates this method of energy acquisition in the iron respiratory electron transport chain, is rusticyanin, a copper containing protein (Djebli *et al*, 1992).

1.1.2 The Genus Leptospirillum

Leptosirillum ferrooxidans belongs to the Nitrospira division of bacteria. It is a gram-negative spiral shaped aerobic, chemoautotropic bacterium that oxidises ferrous iron for energy acquisition. Unlike many other acidophilic bacteria this species cannot use reduced sulphur compounds as a source of energy. This species has recently been found to play a wider role than originally thought, in the generation of acid mine drainage and the dissolution of pyritic substrates. This is due, in part, to this species being less sensitive to inhibition by ferric iron and its ability to oxidise pyritic ores at higher redox potentials than other acidophiles (Bond *et al*, 2000). This species frequently out-competes other iron-oxidisers to become the dominant iron-oxidising organism in such environments (Hallberg & Johnson, 2001 and Rawlings *et al*, 1999)

1.1.3. The Genus Sulfobacillus

This genus is characterised by acidophilic, low G+C gram-positive bacteria that are able to grow heterotrophically on glucose or yeast extract, autotrophically on iron and reduced sulphur compounds or mixotrophically using combinations of these substrates. They are usually non-motile rods and form endospores under conditions of nutrient or other stress.

Only three mesophilic examples of *Sulfobacillus* sp. have been described to date. These include *Sulfobacillus montserratensis* and *Sulfobacillus ambivalens*, which were isolated from a geothermal area of the Island of Montserrat and a detailed characterisation of these strains is currently unpublished. Another mesophile, *Sulfobacillus disulfidoxidans* was found to be more closely related to *Alicyclobacillus* spp. and therefore it was suggested that it be reclassified accordingly (Hallberg & Johnson, 2001).

The rest of the members of this genus are moderate thermophiles with temperature optima between 40°C and 60°C (Hallberg & Johnson, 2001). These include *Sulfobacillus thermosulfidooxidans* with a temperature optimum of 50°C and a maximum of 58°C (Tourova *et al*, 1994) and *Sulfobacillus acidophilus* (Norris *et al*, 1996). Yahya & Johnson (2002) assessed the pyrite bioleaching potential of novel strains of *Sulfobacillus*-like species and found one isolate that could actively leach pyrite at pH 0.8, further highlighting the ability of newly isolated bacteria to challenge the currently accepted boundaries of microbial life.

A number of 16S rDNA sequences have been deposited in the Genbank nucleotide database (NCBI) that relate to *Sulfobacillus* spp. which have not been designated a species name or that remain uncultured. These originate from sources ranging from stirred tank bioleaching operations (Okibe *et al*, 2003) to geothermally heated springs (Atkinson *et al*, 2000), suggesting that this genus is even more diverse than can be appreciated at present.

1.1.4 The Genus Alicyclobacillus

Members of the *Alicyclobacilli* are acidophilic, gram-positive, moderately thermophilic, spore-formers that share a close phylogenetic and physiological similarity with *Sulfobacillus* spp. However, the members of this genus can be distinguished on the basis of the possession of ω-alicyclic fatty acids which are a unique membranous lipid component of *Alicyclobacillus* spp. (Tourova *et al*, 1994).

The characterised members of this genus are reported to be obligate heterotrophs, although results provided in this study provide evidence for autotrophy and mixotrophy as modes of growth of some members of this genus. Members of this genus have been isolated from a diverse range of environments including; *Alicyclobacillus acidophilus*, which was isolated from an acidic beverage (Matsubara *et al*, 2002), *Alicyclobacillus hesperidum* which was isolated from solfataric soils in the Azores (Alburquerque *et al*, 2000) and *Alicyclobacillus cycloheptanicus* which was isolated from herbal tea (Goto *et al*, 2002). There have also been reports of novel Alicyclobacillus-like bacteria isolated from geothermal sites in Yellowstone National Park (Johnson *et al*, 1997) and heterotrophic *Alicyclobacillus* species from geothermal areas of Montserrat (Atkinson *et al*, 2000).

1.1.5 The Genus Sulfolobus

This genus is the most widely characterised of the acidophilic Archea and encompasses both heterotrophic and autotrophic species that are usually thermophilic. They have been isolated from a wide range of environments including hydrothermal vents, geothermal springs and mineral leaching heaps. *Sulfolobus metallicus* is an obligately autotrophic acidophile that acquires energy via the oxidation of reduced sulphur compounds, ferrous iron or metaliferrous sulphidic ores (Huber & Stetter, 1991). *Sulfolobus solfataricus* is an obligate heterotroph with a temperature optimum for growth of 87°C (Norris & Johnson, 1998).

1.1.6 The diversity of acidophiles in acid mine drainage and their role in its formation

Acid mine drainage (AMD) is water containing sulphuric acid, dissolved metals and ferric iron complexes that contaminates rivers and aquifers and is formed by bioleaching of metal sulphides through microbial activity. AMD is produced in areas containing high amounts of sulphidic, metaliferrous minerals that are susceptible to solubilisation by microorganisms. These areas include coal spoilage heaps, mine sites, ore waste heaps and other environments with sulphidic mineral geology. These areas, when exposed to oxygen, water and iron- and sulphur-oxidising bacteria, become acidified due to the metabolic activity of the bacteria. The acid metal-rich drainage water, which is heavily laden with ferric complexes, gives the pollouted area a characteristic orange/rust appearance. This contamination then kills aquatic life in rivers and lakes and makes the contaminated area unsuitable as a source of water or recreation site.

Many different methods have been assessed in order to develop effective remediation technologies that will clean up such contaminated areas. Such methods include; the use of inhibitory compounds to inhibit bioleaching processes (Schippers *et al*, 1996), the use of aerobic reed beds to remove ferric hydroxide and other toxic heavy metals (Wheal Jane Project, 2000), and the use of iron and sulphur reducing bacteria to produce alkalinity to inhibit the growth of leaching microorganisms (Clarke *et al*, 1997 and Johnson, 1995).

However, these areas also support the growth of a highly diverse range of microorganisms and can provide novel isolates for potential use in the biotechnology industry or for bioremediation (Lopez-Archilla & Amils, 2001). Bond et al (2000) and Bond & Banfield (2001) studied the phylogeny of an AMD site and found 16S rDNA sequences that related to a diverse range of bacteria, including recognised and novel members of the Firmicutes, Nitrospira, Thermoplasmes and Proteobacteria divisions. Improved detection and culture techniques may show even higher diversity in these environments in the future.

1.2 Chemoautotrophy in acidophilic bacteria

Chemoautotrophic microorganisms obtain energy for metabolism from inorganic chemicals and rely on the fixation of inorganic carbon for provision of their organic constituents. The microorganisms of interest in this study are chemoautotrophic acidophiles that use iron and reduced sulphur compounds as electron donors for their respiratory processes and subsequent energy production and growth.

The yield of ATP from using inorganic molecules for growth is low compared to the oxidation of organic molecules, moreover, considerable energy is also required to reduce CO_2 to carbohydrate (autotrophy). Therefore, these microorganisms must oxidise a relatively large quantity of inorganic material to grow and reproduce, thus magnifying further their ecological impact. However, chemoautotrophs thrive despite this apparent inefficiency due to them having few serious competitors for their unique energy sources.

Table 1.2 Energy yields from oxidations used by chemoautotrophs. Modified from Prescott *et al*, 1996

Reaction	**ΔG° (kJ/mole)**
$S^\circ + 1\frac{1}{2}\ O_2 + H_2O \longrightarrow H_2SO_4$	-495.80
$S_2O_3^{2-} + 2O_2 + H_2O \longrightarrow 2SO_4^{2-} + 2H^+$	-935.96
$2Fe^{2+} + 2H^+ + \frac{1}{2}\ O_2 \longrightarrow 2Fe^{3+} + H_2O$	-46.86
$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$ (glucose)	-2870.22

Table 1.2 shows that the oxidation of one mole of glucose provides 61.25 times the energy of that liberated by the oxidation of the same amount of ferrous iron. This fact goes some way to explaining the slower growth rates of chemoautotrophic bacteria as compared to heterotrophic bacteria.

1.2.1 Metabolism of pyrite/iron-disulphide (FeS_2)

Pyrite is the sulphide of iron and is the commonest sulphide mineral with widespread occurrence and it is commonly known as 'fools gold' due to its brass-yellow colour. It occurs as an accessory mineral in igneous and sedimentary rocks and is commonly associated with metals such as copper and gold in ore bodies. It is a common substrate of iron and sulphur-oxidising bacteria in nature as it is a more stable form of reduced iron and sulphur than soluble forms at acidic pH. The oxidation of this substrate may cause acid mine drainage contamination when the localised environment is acidified and bacterial activity increases the rate of dissolution of this mineral.

The oxidation of pyrite is a complex process and is not yet fully understood in detail. The dissolution of pyrite involves, firstly the direct oxidation of pyrite (Equation 1.1), the oxidation of ferrous ions thus formed in solution (Equation. 1.2) and then the oxidation of the sulphur yielded by the ferric ions produced (Equations. 1.3 and 1.4), (Morin, 1995).

$$4FeS_2 + 14O_2 + 4H_2O \longrightarrow 4FeSO_4 + 4H_2SO_4 \qquad \textbf{Equation 1.1}$$

$$4FeSO_4 + O_2 + 2H_2SO_4 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad \textbf{Equation 1.2}$$

$$FeS_2 + Fe_2(SO_4)_3 \longrightarrow 3FeSO_4 + 2S^o \qquad \textbf{Equation 1.3}$$

$$2\,S^o + 2H_2O + 3O_2 \longrightarrow 2H_2SO_4 \qquad \textbf{Equation 1.4}$$

Also, a high proportion of the reaction products precipitate into insoluble ferric compounds as outlined in Equations 1.5 and 1.6 below.

$$Fe_2(SO_4)_3 + 6H_2O \longrightarrow 2Fe(OH)_3 + 3H_2SO_4 \qquad \textbf{Equation 1.5}$$

$$Fe(OH)_3 + H_2SO_4 \longrightarrow Fe(OH)(SO_4) + 2H_2O \qquad \textbf{Equation 1.6}$$

Intermediary sulphur compounds such as polythionates are also formed during pyrite degradation and this mechanism is shown in the cyclic pyrite oxidation mechanism shown in Figure 1.1. Some reactions are abiotic and others are catalysed by bacteria.

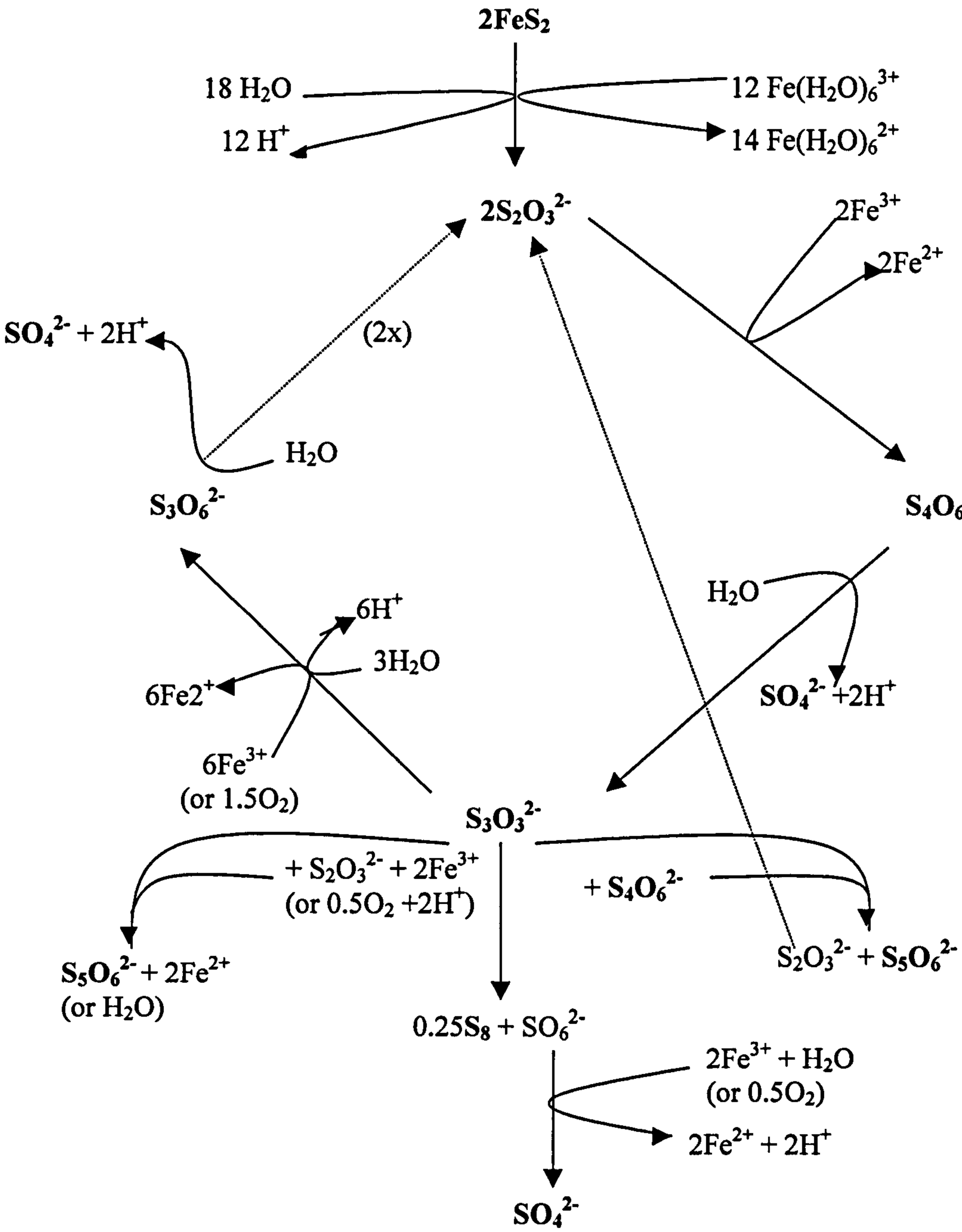


Figure 1.1 Cycle of pyrite degradation. Pyrite is attacked by iron(III) hexahydrate ions. Thiosulphate, as the first intermediary sulphur compound, is degraded via tetrathionate, disulfane-monosulfonic acid, and trithionate to sulphate in the cycle. In side reactions, elemental sulphur, pentathionate, and sulphate occur. Chemical and/or biological oxidation of intermediary sulphur compounds proceeds with iron(III) ions or molecular oxygen as electron acceptor (Schippers *et al*, 1996)

1.2.2 Metabolism of ferrous iron

Under oxygenic conditions ferrous iron is rapidly oxidised to ferric iron in environments of neutral to basic pH, via abiotic chemical reaction. However, under acidic conditions this reaction is slow and therefore ferrous iron is available for acidophilic bacteria to use as an energy source. However, the Fe^{2+} to Fe^{3+} couple yields very little energy and so the energy generated by each oxidation is used to enhance an existing proton gradient that stores the energy.

The oxidation of ferrous iron by chemoautotrophic bacteria is believed to occur outside the cell because the ferric iron produced is very insoluble at the pH of the cytoplasm in acidophiles (Hallberg & Johnson, 2001). A natural proton gradient exists in acidophilic iron-oxidising bacteria because the periplasm is equilibrated with the outside acid environment (~pH 2.0) relative to the pH of the cytoplasm (~ pH 6.0-7.0) (Cox *et al*, 1979).

Cytoplasmic protons are consumed when oxygen accepts electrons (Equation 1.9) derived from the ferrous iron (Equation 1.7 and 1.8) and therefore, as the ferrous iron is oxidised, the proton gradient becomes more extreme and thus acquires more energy. As these protons flow into the cytoplasm from the periplasm (i.e. down the concentration gradient), an ATPase is powered and ATP is produced by the phosphorylation of ADP using inorganic phosphate (P_i). This mechanism is shown in the schematic Figure 1.2

$$4FeSO_4 + O_2 + 2H_2SO_4 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad \textbf{Equation 1.7}$$

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O \qquad \textbf{Equation 1.8}$$

(in periplasm)

$$2H^+ + 2e^- + {}^1/_2O_2 \longrightarrow H_2O \qquad \textbf{Equation 1.9}$$

(in cytoplasm)

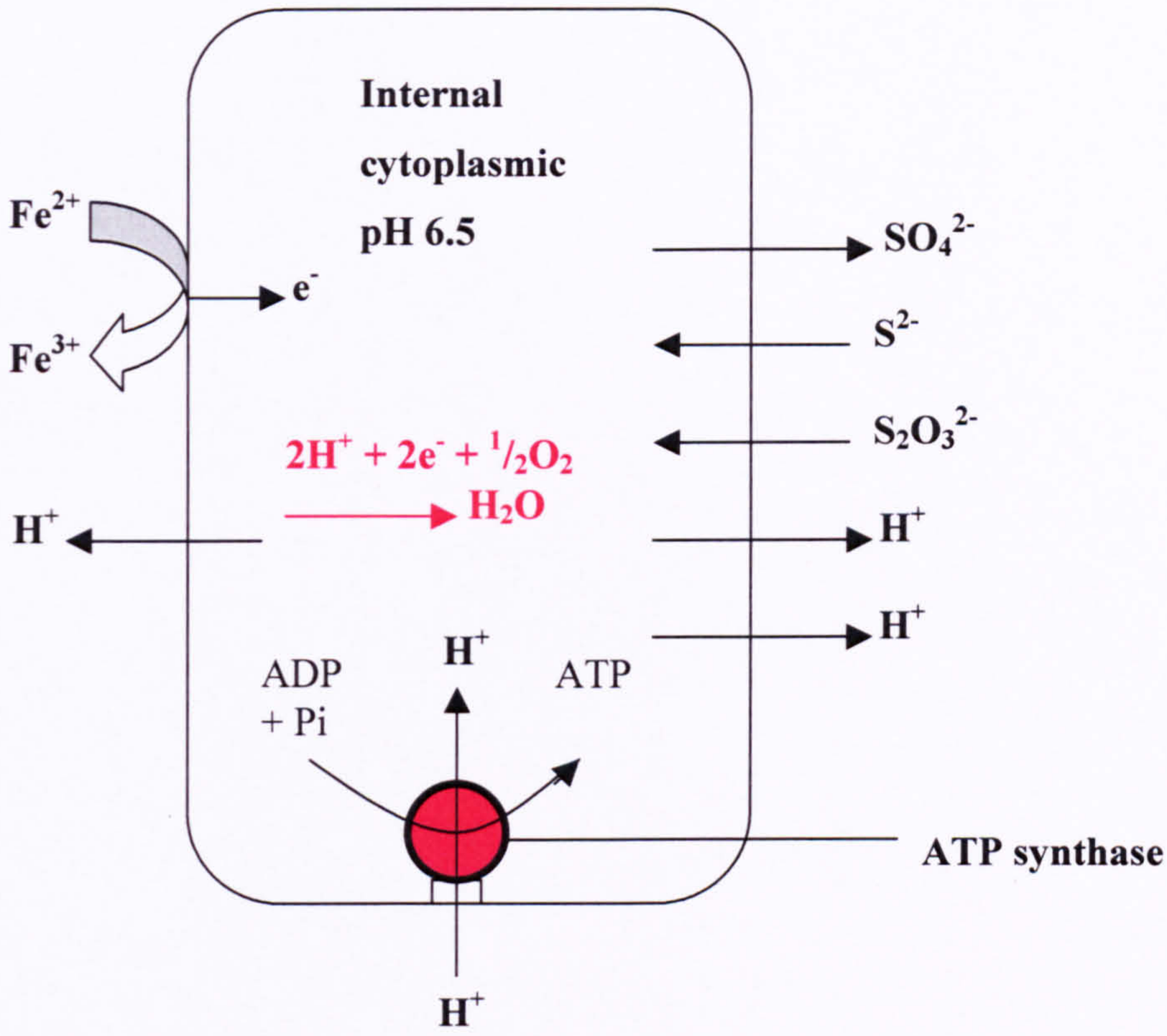


Figure 1.2. A model for the generation of ATP by *At. ferrooxidans* (modified from Rawlings & Woods, 1995). Ferrous iron or reduced sulphur compounds serve as the electron donor and oxygen as the electron acceptor. During respiration, protons are pumped out of the cell and enter via the ATP synthase complex. It is this passage of protons from the outside of the cell, across the cytoplasmic membrane and through the ATP synthase complex that results in the synthesis of ATP (Apel *et al*, 1980). In acidophiles this mechanism is associated with the maintenance of a near neutral intracellular pH.

1.3 The case for the widespread existence of halotolerant, acidophilic bacteria

Iron is the fourth most abundant element in the earth's crust, however, only a small fraction of that total occurs in the reduced form, due to the fact that iron readily oxidises at circumneutral pH and when oxygen is available. Therefore only a small amount is available for growth of iron-oxidising bacteria, except in acidic environments where the oxidation of ferrous iron is biotically mediated.

Iron is highly insoluble in seawater and tends to adhere to organic particles and sink to the bottom of the sea (Kunzig, 2000). Therefore, the amount of available iron in the water column is extremely small, as little as a few parts per trillion, and is thought to be mostly combined in sediments and on available surfaces (Martin *et al*, 1990). However, there have been reports of marine iron-reducing bacteria regenerating ferrous iron, which may serve as a substrate for iron-oxidising bacteria (Lovely, 1991).

The occurrence of chemoautotrophic, acidophilic bacteria in marine environments has been widely noted and these microorganisms have been implicated in biogeochemical cycling of iron and sulphur compounds in the oceans and other high saline environments (Kostka and Luther, 1995 and Tilton *et al*, 1967a and b). However, the growth profiles and physiological responses of these bacteria at elevated salt levels have rarely been described in detail and such bacteria have rarely been isolated to pure culture. This is the case despite widespread interest in their unique metabolic characteristics and potential application in biotechnological processes.

1.3.1 Diversity of sulphur-oxidising bacteria from high salt environments

The first report of isolation of chemoautotrophic acidophilic bacteria from a marine source was as far back as 1902, with the isolation of *Thiobacillus thioparus* from seawater near Naples, which was capable of growth on thiosulphate energy sources (Starkey, 1935). However, the extent of halotolerance was not tested for this strain.

Tilton *et al.* (1967a) described the isolation of 'marine Thiobacilli' from open seawater using membrane filter techniques and thiosulphate enrichment methods. They found very low sulphate-oxidising bacterial numbers, ranging from 0 – 275 per 100 ml. These

bacteria were designated Thiobacilli only on the basis of cell and colony morphology and physiology. They did require seawater in the medium but grew optimally when salinity was reduced to a range of 0.64 % to 2.58 %. With repeated sub-culture these isolates grew progressively better on media of lower and lower salinity but did not grow without at least 10 % seawater (Tilton *et al*, 1967a and b).

Thiobacillus intermedius was isolated from salt marsh sediment in Delaware, USA. This strain grew using various reduced sulphur compounds as substrate and was sensitive to pHs below pH 5.0. The optimal salinity for the growth of this species was found to be 10 gl^{-1}, despite the interstitial water salinity of the isolation site being 20 gl^{-1}. The authors of the study noted that this species of bacteria probably grows at its maximum rate only rarely, as its natural environment conditions are not optimal for growth (Smith & Finazzo, 1981).

The aforementioned reports of chemoautotrophic bacteria from areas of elevated salt, were all isolated and maintained on reduced sulphur compounds, and no reports of iron-oxidation by halotolerant acidophiles were found before 1984.

1.3.2 Diversity of iron-oxidising bacteria from high salt environments

Cameron *et al.* (1984) described iron-oxidising bacteria that were isolated from a tidal estuarine environment but did not grow at high salinity. Iron–oxidation by these bacteria was completely inhibited by 3 % (w/v) NaCl and increased lag periods were observed during growth in media containing levels of NaCl above 1.5 % (w/v). The protective effect of clay minerals (montmorillonite) against salt toxicity to these bacteria was also demonstrated. This protective effect may be due to the high cation exchange capacity of this mineral (Stotszky, 1980).

Huber and Stetter (1989) described a new species of 'metal-mobilising' bacteria isolated from a marine geothermal field. The most extensively studied of their isolates was designated *Thiobacillus prosperus*, an aerobic, autotrophic, gram negative, motile rod. This bacterium was isolated from sediments and seawater samples using a saline medium with a mixed ore sample as substrate. It was thought to be a member of the

genus *Thiobacillus* due to its ability to oxidise sulphur compounds and sulphidic ores to sulphate.

T. prosperus was found to grow at NaCl concentrations of up to 3.5% and has often been referred to as a halophilic microorganism (Hallberg and Johnson, 2001). However, it was found that optimum growth rates were obtained in medium with no added NaCl (and the optimum salinity for growth was found to be 20% that of seawater, 6 gl^{-1} NaCl, when grown on pyrite in this study) and therefore this bacterium should be referred to as halotolerant. *T. prosperus* grows in medium with a pH between 1.0 and 4.5 with an optimum around pH 2.0 The authors suggested that this bacterial strain might be of potential in the leaching of metallic ores, due to its high levels of resistance to cobalt, nickel and zinc ions and its ability to grow on ore mixtures.

Another environment where salt tolerant chemoautotrophs have been implicated in biogeochemical cycling is in hypersaline lakes. *Thiobacillus halophilus* is an obligately chemolithotrophic, halophilic bacterium from an Australian hypersaline lake. This bacterium grows on reduced sulphur compounds and shows optimal growth at 0.8 – 1.0 M NaCl (46.72 gl^{-1} – 58.5 gl^{-1} NaCl) but can tolerate up to 4 M (234 gl^{-1}) NaCl. However, this bacterium is neutrophilic, exhibiting optimum growth at pH 7.0 – 7.3 and does not survive at the low pH values at which acidophiles thrive (Wood & Kelly, 1991 and Wood *et al*, 1991).

There have also been widespread reports of bacterially mediated corrosion of iron containing structures in the marine environment (Edyvean & Videla, 1991, Hamilton, 1995 and Herdendorf, 1995). Some studies describe the occurrence of rust-coloured stalictites caused by iron-oxidising bacteria found on steel and other iron structures on shipwrecks (Herdendorf, 1995). These structures were given the name 'rusticles' (Figure 1.3 shows a picture of rusticles) and these were defined as 'very reddish-brown stalactites of rust, hanging down as much as several feet, caused by iron-eating bacteria' (Ballard, 1986, 1987). Rusticles were found to contain an abundance of iron-oxidising rod-shaped bacteria and some were identified as iron-oxidising species of the genera *Leptothrix* and *Siderocapsa* (Herdendorf, 1995).

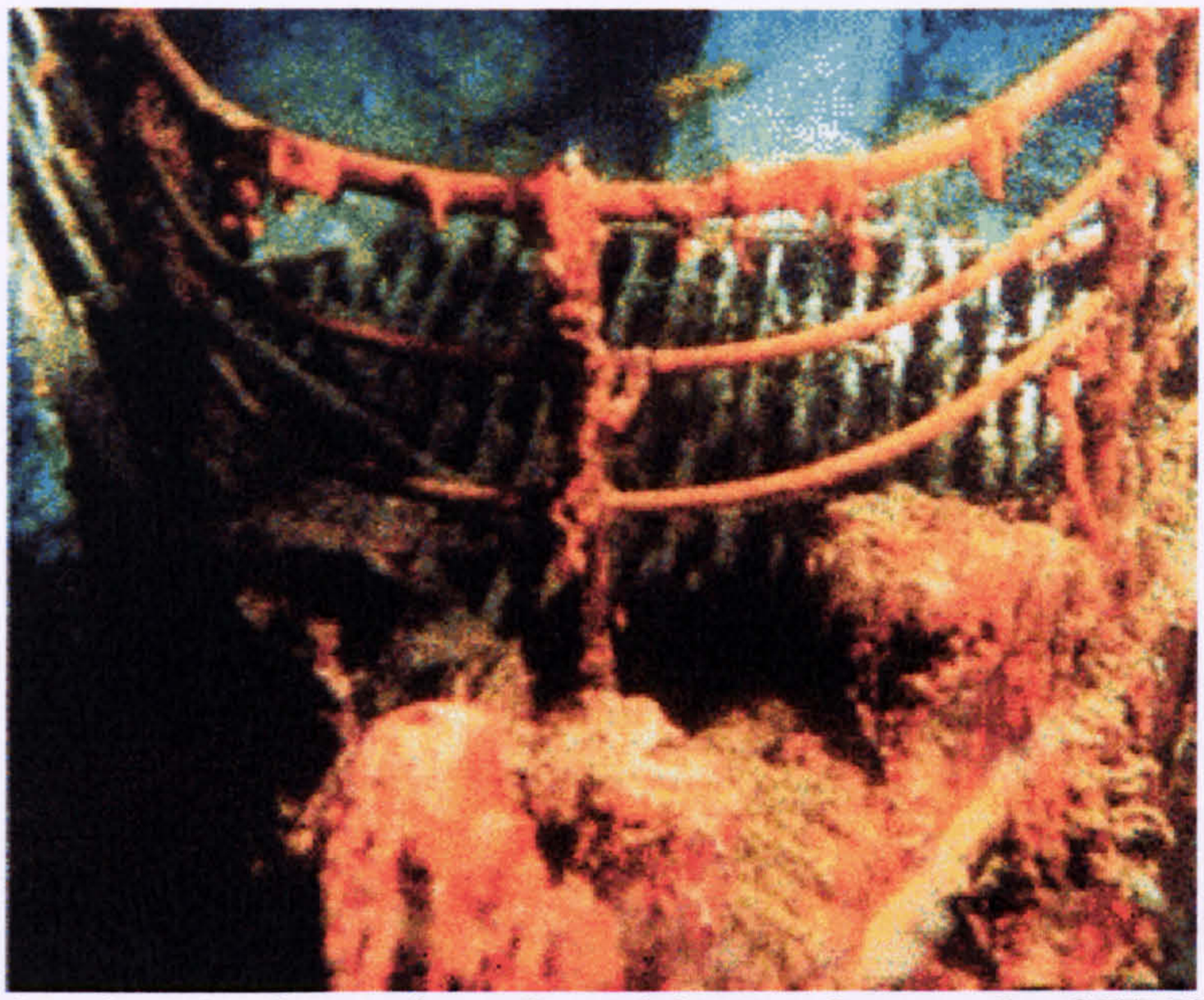


Figure 1.3 showing the formation of rusticles on the wreck of HMS Titanic (from the Chemical Institute of Canada; www.chemisnt.ca/ncw/articles/1994.titanic_e.html)

Hydrothermal vents provide a major source of iron and sulphur in the deep sea. They occur in tectonically active areas of the ocean floor, where cracks allow seawater to mix with molten magma. The heated seawater leaches minerals and heavy metals and the hydrothermal fluids are then emitted into the cold seawater and this produces mineral precipitation in the form of tall mineral chimneys (smokers). The hydrothermal fluids are acidic, reduced and enriched with heavy metals, methane and hydrogen sulphide gas (Prieur, 1997). Figure 1.4 shows a 'black smoker' from a hydrothermal vent system..

Hydrothermal vents support ecosystems that are almost entirely independent of solar energy. The systems, although small in size, are diverse metabolically, physiologically and taxonomically and rely on the growth of chemosynthetic microorganisms for primary production and support of higher life (Eberhard *et al*, 1995). It was calculated by Wirsen *et al* (1986) that approximately 79% of the total bacteria around deep-sea hydrothermal vents in the east Pacific Ocean were represented by chemotrophic sulphur oxidising bacteria. Figure 1.5 shows a schematic of the biochemical interface in black smoker environments. Demming & Barross (1983) suggested that these environments could represent 'windows to a subsurface biosphere'; because this extreme environment was found to support life, the existing limits for life have been further broadened and it is therefore probable that life exists deeper in the earth's crust than is currently believed.

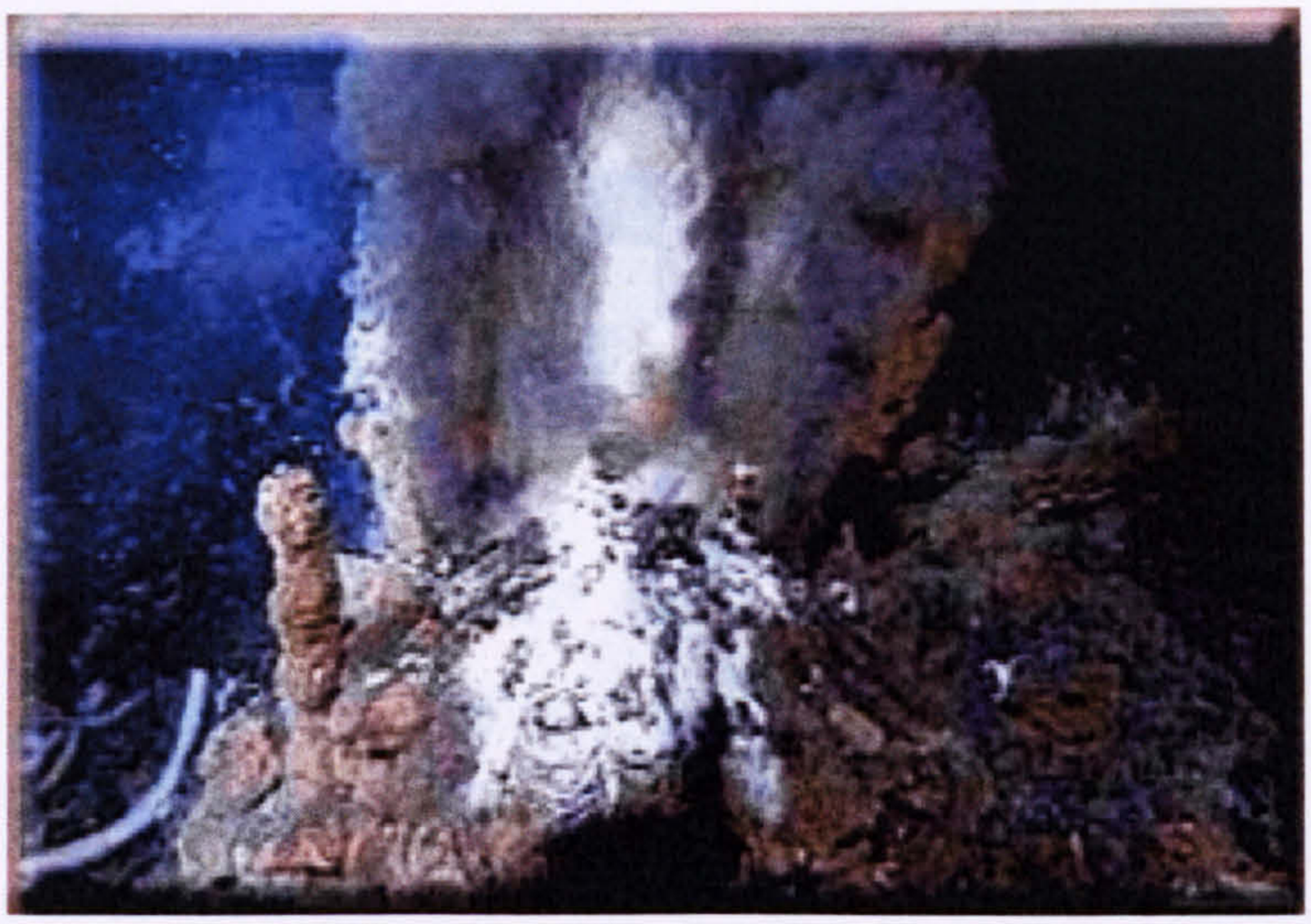


Figure 1.4. A black smoker from a hydrothermal vent system in the Pacific (re-printed with kind permission from The Office of Naval Research, US; www.onr.navy.mil)

Chemoautotrophic bacteria, that oxidise various sulphur and iron-containing compounds, have been detected from many marine hydrothermal sources (Eberhard *et al*, 1995; Gugliandolo & Maugeri, 1993; Harmsem *et al*, 1997; Prieur, 1997 and Verati *et al*, 1999). The sulphur and iron rich properties of this habitat make it an ideal environment for chemoautotrophic primary production. Much research has been carried out into the use of these organisms in high temperature leaching of metals from sulphidic ores (Norris *et al*, 1996), however the growth physiology of these microorganisms have not been assessed at high salinity.

Chemolithotrophic, gram negative rod shaped bacteria were isolated from a shallow hydrothermal vent environment in Vulcano, Italy. The sulphur-oxidising bacteria were found to grow with thiosulphate as the sole energy source (Gugliondolo & Maugeri, 1993). Analysis of the 16S rDNA from acidic sites from the same hydrothermal vent system at Vulcano was reported by Simmons & Norris (2002). The authors found sequences related to *T. prosperus* (which had been previously isolated from the same site, Huber & Stetter, 1989) and *Acidithiobacillus* sp. The 16S rDNA was isolated directly from the samples to investigate the diversity of hydrothermal vent systems without the selective bias observed with the use of selective culture techniques. However, the molecular methods and oligonucleotides used to amplify the 16S rDNA regions may have selected for certain organisms. Thermophilic Archea similar to *Sulfolobus metallicus* were also isolated from the hot vent samples during the same investigation. These strains could be cultured using elemental sulphur, pyrite and yeast

extract as substrates but growth of these enrichment cultures could not be maintained in the presence of 3% salt (Simmons & Norris, 2002).

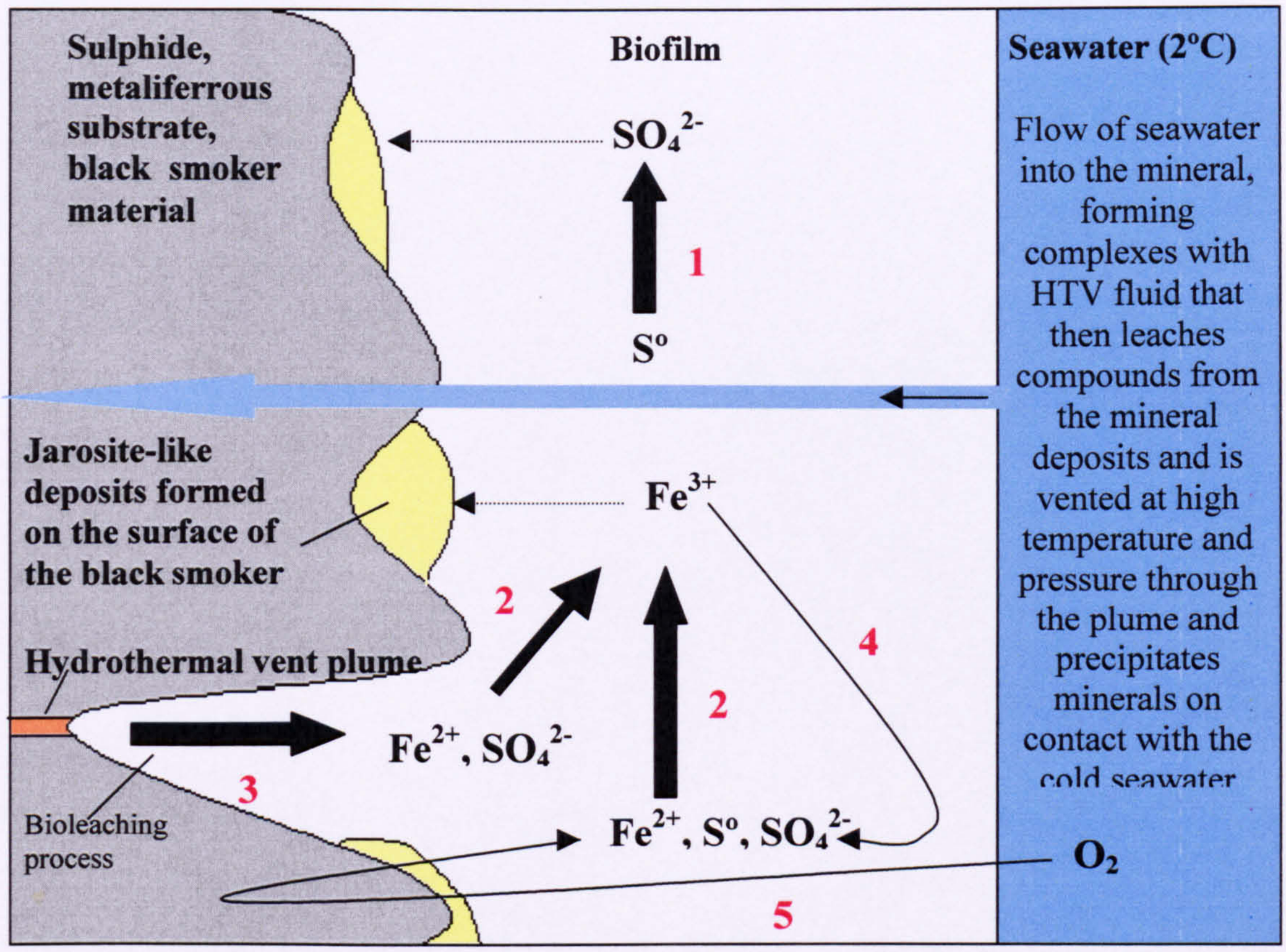


Figure 1.5. Schematic of the bio-chemical interface in black-smoker, hydrothermal environments (Modified from Verati *et al*, 1999).

Key:

Oxidation of sulphur by bacteria

1: $S^o + {}^3/_2O_2 + H_2O \longrightarrow SO_4^{2-} + 2H^+$

Oxidation of pyrite by bacteria

2: $2Fe^{2+} + 0.5O_2 + 2H^+ \longrightarrow 2Fe^{3+} + H_2O$

3: $FeS_2 + 3.5O_2 + H_2O \longrightarrow Fe^{2+} + 2H^+ + 2SO_4^{2-}$

Oxidation of the mineral by dissolved O_2 and Fe^{3+}

4: $14Fe^{3+} + FeS_2 + 8H_2O \longrightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$ with $SO_4^{2-} \rightarrow (S^o)$

5: $FeS^{2+}\ 2H^+ + 0.5O_2 \longrightarrow Fe^{2+} + 2(S^o) + H_2O$

Bacterial process

Chemical process

Jarosite precipitation

Flow of seawater through cracks in the mineral, forming complexes with HTV fluid that then leaches compounds from the mineral deposits and is vented at high temperature and pressure through the plume and precipitates minerals on contact with the cold seawater.

A marine, halotolerant, iron-oxidising acidophilic bacterium that obligately required NaCl for growth was isolated from open seawater in Japan. Strain KU2-11 was enriched using ferrous sulphate liquid medium with 2.7% NaCl. This strain is a gram-negative rod-shaped bacterium with an optimum growth temperature of 30°C and pH of 2.0 (Kamimura *et al*, 2001). Physiologically, strain KU2-11 was found to be very similar to *At. ferrooxidans*, however, phylogenetic analysis showed that this strain is distinct from *At. ferrooxidans* and exhibited a closer relationship to *Acidiphilium multivorum* and *T. novellus*. However, the physiological characteristics of this halotolerant strain were very different to these phylogenetically close bacteria.

The optimum NaCl concentration for growth of KU2-11 was 2% (approximately 66% that of average seawater salinity). The authors noted that total final biomass of their strain when grown in medium containing NaCl was much less than that of *At. ferrooxidans* a (terrestrial bacterium) in medium with no added salt. Also, iron-oxidation rates under optimum growth conditions were much less than those of iron-oxidising terrestrial strains under their optimal conditions

Following the isolation of KU2-11, a further five hundred marine samples were used in enrichment attempts in order to isolate iron-oxidising acidiphilic bacteria. However, these attempts were all unsuccessful (Kamimura, Okayama University, Japan, personal communication, 2002).

During their study, Kamimura's group also demonstrated the sensitivity of four different strains of *At. ferrooxidans* to increasing levels of NaCl. This result was in concurrence with other similar studies on the toxicity of NaCl on the growth of *At. ferrooxidans* (Kamimura *et al*, 2001; Lawson *et al*, 1995; Lazaroff, 1962 and Razzell & Trussel 1963)

As part of a program to develop bioremediation technology for metal contaminated harbour sediments, a search for salt-tolerant iron-oxidising bacteria was undertaken by researchers at the University of New South Wales, Australia in collaboration with ANSTO (Australian Nuclear Science Technology Organisation). Holden *et al* 1999 reported the isolation of such bacteria from Sydney Harbour sediment using artificial seawater containing 1% salts. All three of their isolated strains exhibited optimal

growth and iron oxidation in medium containing 1% NaCl and doubling times increased proportionally as salt concentration increased above 1%.

The isolated bacteria were capable of mixotrophic growth on ferrous sulphate and yeast extract and heterotrophic growth on yeast extract, whereas, autotrophic growth by these strains was not detected. The optimal temperature for growth of these strains was not reported but the maximum temperature for growth was 44°C. Sequence analysis of the 16S rDNA showed that these bacteria bore the greatest resemblance to *Sulfobacillus* sp. The metal leaching abilities of these strains were also reported and are discussed in Section 1.5.1. Table 1.3 details some of the iron- and sulphur-oxidising bacteria that have been isolated from high salt environments.

Table 1.3 Salt-tolerant iron- and sulphur-oxidising bacteria isolated from high salt environments

Bacterial name or isolate	Location and environment of origin	Substrates for growth	Optimum salinity	Salinity range	pH	Temperature	Other details	Reference
T. prosperus	Shallow hydrothermal vents of Vulcano, Italy	Ferrous iron, S°, pyrite and metal sulphides	0 %	0 - 3.5%	Optimum: pH 2.0 Range: pH 1 - 4.5	Optimum: 37°C Range: 23 - 41°C	Gram –ve, motile, rod shaped bacterium, 4 µm long by 0.2-0.4 µm wide, G+C content 61-64 mol %	Huber & Stetter, 1989
KU2-11	Sea water (Seto Inland Sea, Japan). Enriched at 2.7% NaCl	Ferrous iron, S°	2%	1 - 4% Obligately requires NaCl for growth.	Optimum: pH 2.0 Range: not reported	Optimum: 30°C Range: not reported	Gram –ve, motile, rod shaped, 0.4 µm wide by 1.1 µm long, G+C content 59 mol %.	Kamimura *et al*, 2001
T. intermedius	Salt marsh sediment with an interstitial water salinity of 3%	Thiosulphate, FeS, S° and complex organic medium	1%	0 - 4% NaCl, (growth slow at extremes)	Optimum pH 6.0 Sensitive to pHs under 5.0, slow growth down to pH 2.0	Optimum: 25°C Range: not reported	Gram –ve, motile rods, 0.5 µm wide by 1.0 long µm, G+C content: not reported	Smith & Finazzo, 1981
Isolate T1	Mud samples from the tidal range of River Tamar, UK	Ferrous iron	0%	0 – 1.5%	Optimum: not reported Range: not reported	Optimum: 20°C Range: not reported	Gram –ve rods, 0.8 µm wide by 1.5 µm long, G+C content: not reported	Cameron *et al*, 1984
Strains A2-8, A19-22, A20-24	Sediment from Sydney Harbour, Australia	Ferrous sulphate, pyrite,metal sulphides, yeast extract	1%	0.5 – 5%	Optimum: not reported Range: pH 1.6-4.5	Optimum: 44°C Range: not reported	Gram +ve rods, 0.2-0.5 wide µm by 0.8-3.0 µm long, G+C content: not reported	Holden *et al*, 1999
WH-2	Open seawater from the Atlantic ocean	Thiosulphate and S°	1.5%	0.64% - 2.58% NaCl	Optimum: not reported Range: pH 2.1 –5.6	Optimum: 30°C Range: not reported	Gram –ve motile rod-shaped bacterium, 1 – 3 µm long, G+C content: not reported	Tilton *et al*,1967a & b
Strain SH	Open seawater	Tetrathionate and S°	Not reported	Obligate requirement for salts	Optimum: pH 4 Range: not reported	Optimum: 30°C Range: not reported	Gram –ve motile rod-shaped bacterium, G+C content 46 mol %	Kamimura *et al*, 2003

1.4 Biomining: theory and industrial practices

The mineral mining industry is continually looking for new ways to enhance their existing technology and one such way is the incorporation of biotechnology into the extraction of metals from mineral ores. Traditional extraction methods such as solvent extraction, pressure leaching and electrowinning are cost effective for extraction of commercial metals from high-grade ores, but it is not economic or efficient to use these methods for metal extraction form low-grade ore bodies, or low-grade waste ore. Legislation also dictates that existing technology must be made more environmentally co-operative, and complying with this further adds to the capital costs involved in existing mining technology.

As a result of this demand, the use of acidophilic microorganisms has been recognised as a viable option for treatment of low-grade ores with a view to metal extraction (biomining). The indigenous physiological capacity of chemoautotrophic bacteria can be harnessed to leach these metals.

Biomining is the use of microorganisms in the recovery of industrially important metals and this term encompasses both bioleaching and biooxidation. These terms are sometimes used interchangeably, however, bioleaching should be used to refer to the conversion of an insoluble metal into a soluble form (e.g. from a metal sulphide into a metal sulphate), thereby extracting the target metal into water. Biooxidation refers to processes in which the recovery of a metal is enhanced by microbial decomposition of the mineral but the target metal is not solubilised and subsequently requires the use of traditional methods for recovery of this metal (Rawlings, 2002; Schippers & Sand, 1999).

1.4.1 Mechanism of bacterially mediated metal extraction

The bioleaching process is a complex system involving both biotic and abiotic factors. These biological and chemically mediated processes are inextricably linked and depend upon each other for the success of leaching systems. There are a number of different schools of thought regarding the mechanisms involved in biomining processes, however, it is increasingly becoming accepted that two biological systems contribute to the leaching of metals and oxidation of the mineral sulphides. These systems are

commonly referred to as the direct (contact) and indirect (non-contact) mechanisms. Figure 1.6 shows an overview of the proposed mechanisms of pyrite bioleaching.

The direct mechanism involves the attachment of the bacteria to the mineral particle enhancing the rate of mineral dissolution by the use of iron and sulphur compounds for the energy acquisition within the bacterial cell membrane via enzymatic reactions (Rawlings, 2002; Sand *et al*, 2001 and Silverman & Ehrlich, 1964). Equations 1.10 and 1.11 outline the chemical and bacterially mediated reactions occurring during the direct bioleaching mechanism. The indirect leaching mechanism involves the regeneration of ferric iron ions from ferrous iron by oxidation mediated by the non-attached bacteria in the lixivant (Equation 1.12). Ferric iron then chemically attacks the mineral sulphide, resulting in the dissolution of the mineral and release of ferrous iron and sulphur moieties (Equation 1.13 and 1.14). These can then be re-used by the bacteria to regenerate the ferric iron and continuation of the cycle (Rawlings, 2002; Sand *et al*, 2001).

Direct mechanism

$$FeS_2 + 3.5O_2 + H_2O \longrightarrow Fe^{2+} + H^+ + 2SO_4^{2-} \qquad \textbf{Equation 1.10}$$

$$2Fe^{2+} + 0.5O_2 + 2H^+ \longrightarrow 2Fe^{3+} + H_2O \qquad \textbf{Equation 1.11}$$

Indirect mechanism (M = metal)

$$FeS_2 + 14Fe^{3+} + 8H_2O \longrightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-} \qquad \textbf{Equation 1.12}$$

$$MS + 2Fe^{3+} \longrightarrow M^{2+} + S^o + 2Fe^{2+} \qquad \textbf{Equation 1.13}$$

$$S^o + 1.5O_2 + H_2O \longrightarrow 2H^+ + SO_4^{2-} \qquad \textbf{Equation 1.14}$$

(Modified from Sand *et al*, 2001)

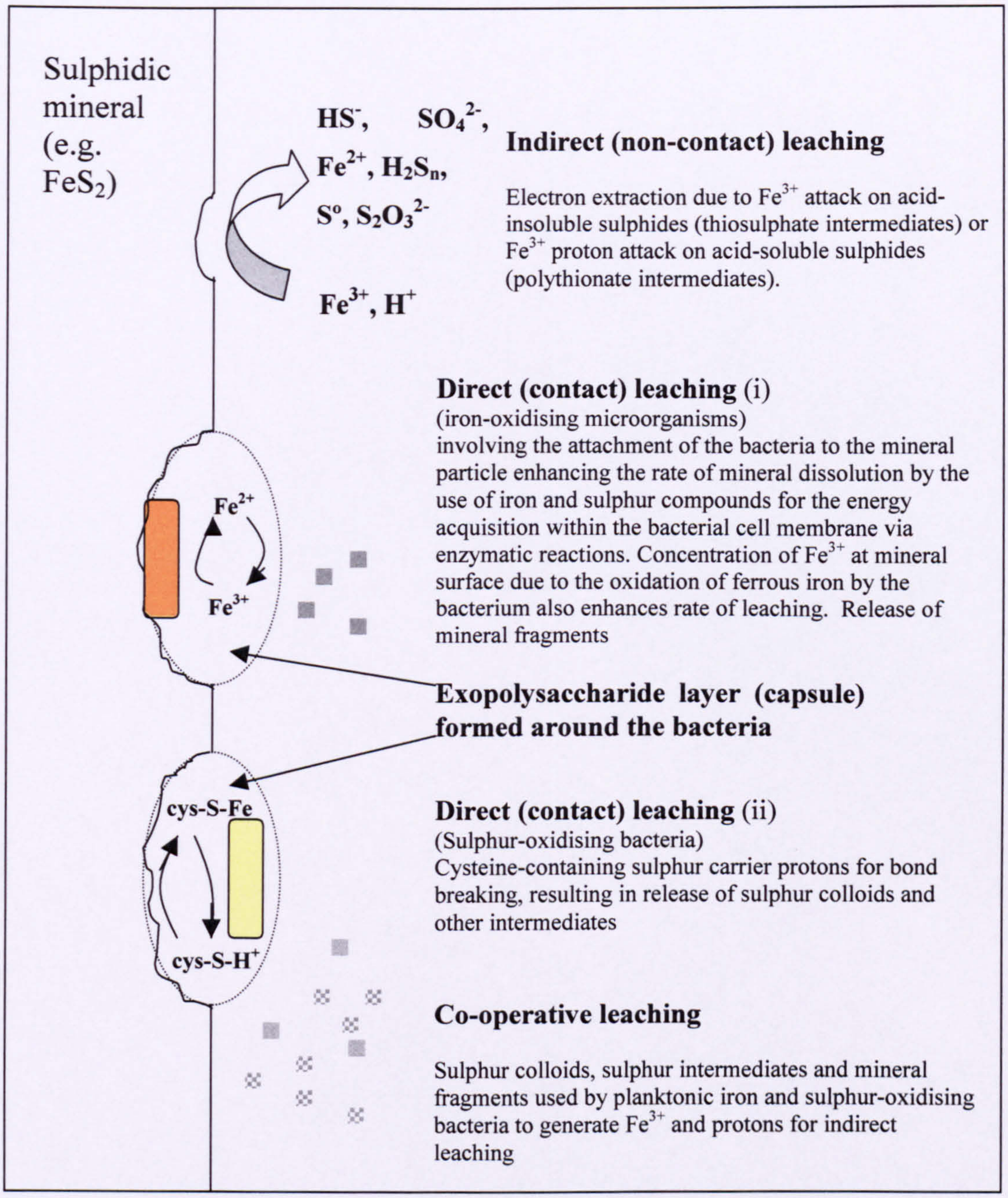


Figure 1.6. Schematic diagram illustrating the proposed mechanisms of pyrite bioleaching (modified from Rawlings, 2002 and Tributsch, 2001). The diagram shows the direct (contact), indirect (non-contact) and the co-operative mechanisms of bioleaching.

Iron-oxidising bacterium

Sulphur-oxidising bacterium

Sulphur colloids/intermediates

Mineral fragments

However, although these are the widely considered views of the mechanisms, there are different ideas on how the bacteria carry out these reactions. One view suggests that ferric iron is the only chemical agent responsible for dissolving the ore. This theory also proposes that the bacteria, in conjunction with their excreted exopolysaccharide glycocalyx, are only responsible for the regeneration of this agent and resulting concentration of it at the mineral/water interface (Sand *et al*, 2001). The proposed mechanism encompasses both the thiosulphate and polysulphide reactions as outlined in Figure 1.7.

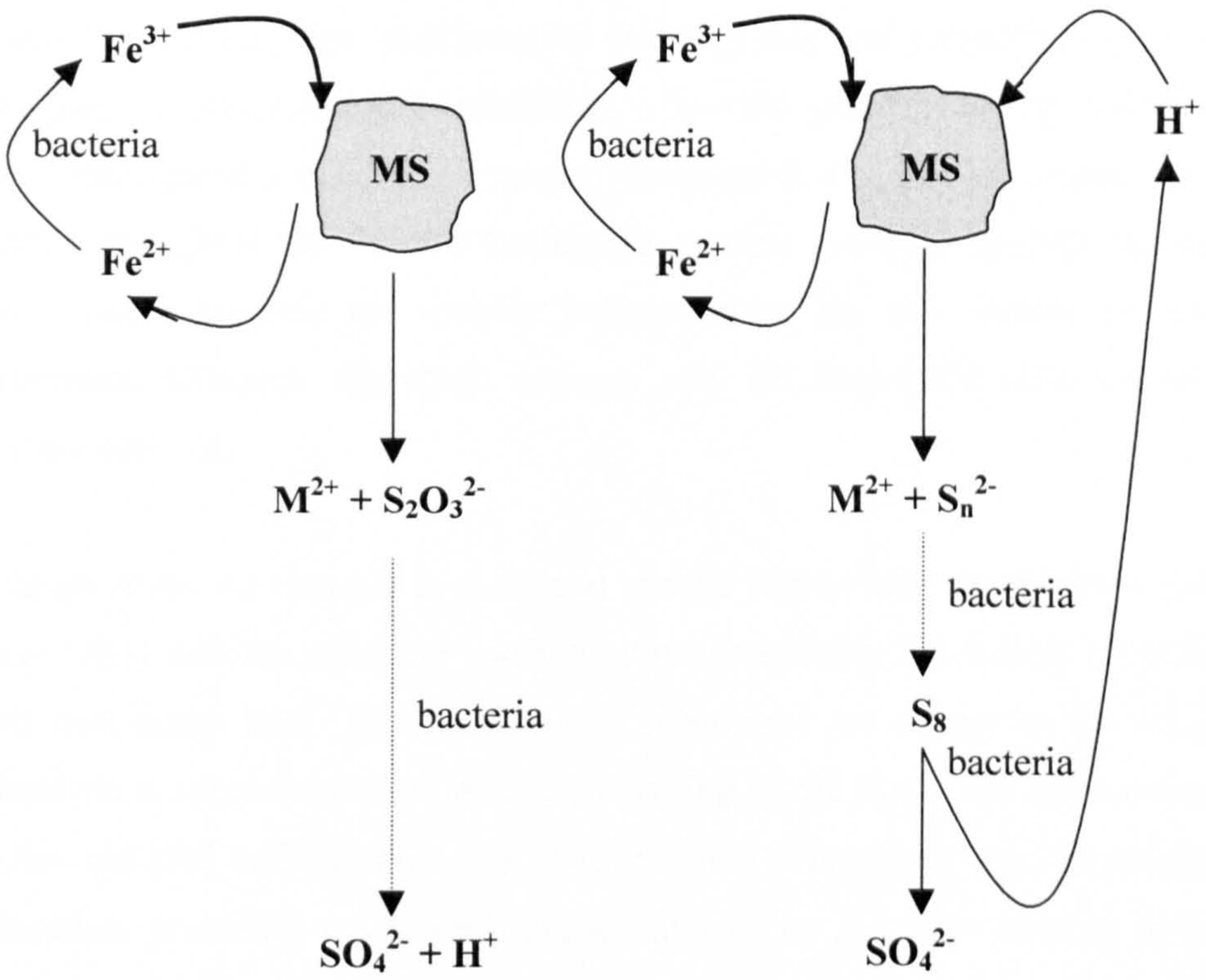


Figure 1.7 Scheme of thiosulphate and polysulphide mechanism on (bio)leaching of metal sulphide (modified from Schippers and Sand 1999).

Key : MS – metal sulphide, M^{2+} - metal ion, $S_2O_3^{2-}$ - thiosulphate, S_n^{2-} - polysulphide with chain length n, S_8 – elemental sulphur, bacteria – enzymatic reaction by chemoautotrophic bacteria.

1.4.2 Types of biomining operations

There are several different processes that utilise bacteria in the biomining industry and new processes are being patented regularly. The main processes that are currently industrially used are outlined in this section.

Dump and heap leaching involve the deposition of low-grade ores (which are usually waste products or concentrates from metal extraction from high-grade ores) on an impermeable slope. The top of the heap is irrigated by spraying a leach solution on the surface. This solution contains sulphuric acid, which serves to reduce the pH to between 3.0 and 1.5, thus increasing the leaching rate and preventing ferric salts from building up by increasing their solubility. Bacteria grow in the top metre of a dump heap or throughout a heap leach system (heap leach systems are usually aerated and irrigated throughout the layers, facilitating aerobic bacterial growth throughout the heap). These bacteria are usually indigenous to the ore sample or surrounding environment, although the leach lixivant may be inoculated with certain defined bacterial consortia.

The target metal e.g. copper is dissolved via the bioleaching mechanisms (outlined in section 1.4.1) and the resulting leach solution containing this copper is collected and passed over scrap iron. The iron is then substituted for copper in the soluble metal sulphate via a simple chemical reaction resulting in the liberation of elemental copper (reaction outlined in Equation 1.15). The resulting copper may then be precipitated via cementation processes or solvent extraction can be used to recover a more pure concentrated copper product. Figure 1.8 shows a schematic of a typical dump leach system.

$$CuSO_4 + Fe^o \longrightarrow FeSO_4 + Cu^o \qquad \textbf{Equation 1.15}$$

This process has been successfully employed in several industrial leach operations. These include the Quebrada Blanca heap leach operation in northern Chile which processes 17,300 tonnes per day of sulphide ore to produce 206 tonnes per day of copper (cathode grade) (Brierley & Brierley, 1999 and Schnell, 1997). The Zaldivar operation in Chile operates a dump leach system with a processing rate of 20,000 tonnes

per day and as far back as the 1970's Kennecott Copper operated a heap leach system with an output of 200 tonnes per day (Bosecker, 1997).

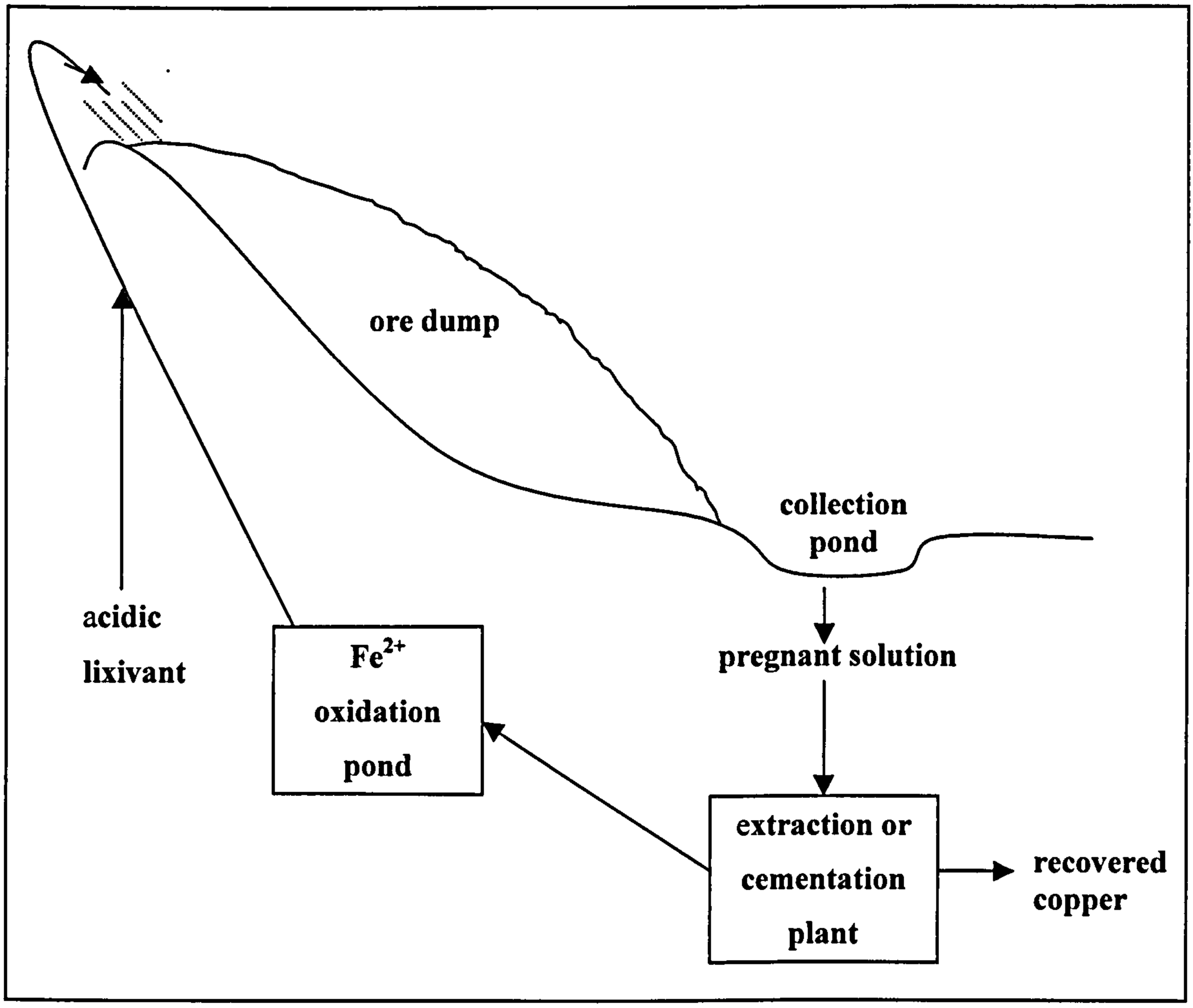
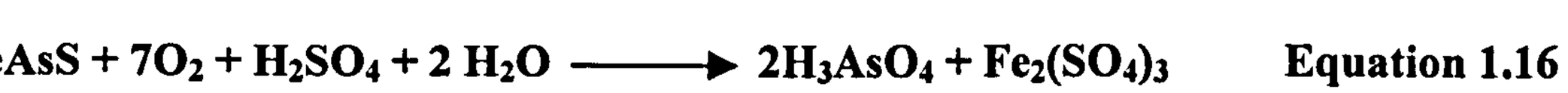


Figure 1.8 Components of a copper ore dump leach operation. Modified from Olsen & Kelly (1986). The leachate solution with dissolved copper from the ore pile runs into the collection pond and from there undergoes cementation or further extraction processes to recover the copper. The barren leachate may then recycled by the oxidation of Fe^{2+} by bacteria in a oxidation pond, thus regenerating Fe^{3+}, and this is then added to fresh lixivant and used to irrigate the ore pile. The bioleaching of copper occurs in the ore pile and is mediated by iron-oxidising bacteria.

Low overall capital costs are associated with these methods of bioleaching, with the only substantial cost being the addition of sulphuric acid and purchase of scrap iron for cementation. These costs are much lower than chemical and/or physical extraction methods, and waste products may be re-used as leach-liquor, thereby making this process more environmentally friendly when these operations are properly maintained and reclaimed at the end of the life of the mine.

Tank or bioreactor leaching is usually used for the biooxidation of recalcitrant/refractory gold ores (Bosecker, 1997) and can be piloted in the lab in shakeflasks that can then be scaled up to larger batch reactors in oxidation plants. Gold in recalcitrant ore is encased in a matrix mainly consisting of arsenopyrite/pyrite and cannot be efficiently recovered using pressure leaching or direct cyanidation treatment alone. Fortunately, bacteria can be used to decompose the mineral sulphide matrix (Equation 1.16) to make the mineral amenable to efficient metal recovery by cyanidation (Bosecker, 1997, Hayward *et al*, 1997; Nestor *et al*, 2001; Rawlings, 1998 Ruitenberg *et al*, 1999).



Equation 1.16

Examples of such processes include the Biox® process (Dew *et al*, 1997) but production rates and process advantages are rarely reported, due to competition from industrial competitors. However, reports of the capital costs using this technology being two-fold lower than roasting or smelting have been noted (Bosecker, 1997).

Although running and maintenance costs are high with bioreactor operations (including bioreactor manufacture, heating and agitation costs), the improvement in gold recovery offsets the high capital costs involved, which once again are lower than using chemical and physical methods alone to obtain the same gold production. However, the design of such a bioreactor must be considered very carefully with regard to microbial-consortia selection, agitation method and speed, materials used and temperature control, as these all effect the rate of biooxidation and cost of operation (Hayward *et al*, 1997 and Spencer, 2001). For example, the selection of microbial-consortia that are very tolerant to elevated metal levels such as arsenic must be considered.

1.5 The case for biomining at high salinity

The mineral industry is constantly looking for technology that is more efficient and economic as well as being less environmentally damaging due to economic competition and environmental legislation. Water is a valuable commodity in the biomining industry and large quantities are needed for leaching lixivant, cooling fluid or flotation liquid. High capital costs are involved in the purchase of water and the transport logistics of water to biomining operations. The use of seawater in such processes would greatly benefit operations that are closer to marine rather than to freshwater sources. Also, many mine sites are contaminated with salts from nearby saltpetre deposits, or have an available source of brackish borehole water that may be a potential source of lixivant. However, it was noted by Dew *et al* (1997) that more expensive stainless steel would be required for the use of high chloride process water in these systems, due to the high corrosive effect of chloride ions on metal containing materials.

Budden & Spencer (1991) reported a chloride content of borehole waters of between 12.7 – 25.7 gl^{-1} and Weston *et al* (1994) reported a high level of 110 gl^{-1} chloride in borehole water. Analysis of Lihir Island gold operation borehole water revealed a chloride content of 34 gl^{-1} (Rio Tinto Technical analysis report, this study). The analysis of this geothermal well water is outlined in Table 1.4. The total dissolved solids in this well water sample was found to be 117.3 gl^{-1}. Chloride potentially enhances the rate of leaching of metaliferrous ores by acting as a corrosive agent and may give a significant process advantage if it was used in biomining operations.

However, these saline water sources are rarely assessed for biomining processes due to the inhibition of growth of the bacterial consortia traditionally used in such operations, because of their high sensitivity to elevated salt levels. Various limits for the growth of *At. ferrooxidans* have been reported (Deveci, 2002; Huber & Stetter, 1989; Lawson *et al*, 1995 and Leong *et al*, 1995). However, the consensus seems to be that NaCl concentrations above 1% have a detrimental or inhibitory effect on the growth of this bacterial species. The removal of these toxic levels of salts is not cost effective.

Table 1.4 Analysis of geothermal well water from the Lihir Mining Company (Rio Tinto technical report)

Chemical species	Concentration (g l^{-1})
chloride	34
potassium	8.89
sodium	43.3
silicon	0.0775
calcium	0.00277
magnesium	0.00021

However, despite the potential advantages of the application of such high salt biomining technology, research into this subject has been scarce and those reports found in the literature have rarely compared leaching rates with traditional characterised bioleaching strains.

1.5.1 Bioleaching and biooxidation attempts at high salinity

Huber and Stetter (1989) found that halotolerant iron-oxidising isolates (including *T. prosperus*) showed extraction values of up to 100% for uranium, 40 % of zinc and up to 8 % of the total copper in a mixed ore sample. This result was produced at a salinity of 3.5% NaCl after 28 days incubation at 37°C and 3% ore load (w/v) and the same rates were observed in medium without added salt. Leong *et al* (1993) reported 90 – 95% copper extraction from chalcocite by a mixed bacterial culture in the presence of 8 gl^{-1} chloride after 20 days leaching.

During the assessment of the effect of chloride on the BIOX® process it was noted that the bacterial consortia used showed little growth inhibition at concentrations of chloride in the range of 0 – 5 gl^{-1}. However, the rate of iron oxidation during this process decreased from 80% oxidation when no salt was added to 55% oxidation after 24 hours at concentrations of 7 gl^{-1} chloride (0.7%). Above 19 gl^{-1} complete inhibition of bacterial activity was caused (Dew *et al*, 1997).

In addition, some of the reports of bioleaching attempts at elevated salinity describe the precipitation of jarosites at high chloride concentrations (Dew *et al*, 1997; Deveci, 2002; Leong *et al*, 1993). Jarosite formation is the result of the chemical reaction between ferric iron, sulphate and soluble potassium (outlined in Equations 1.16 and 1.17) and occurs as brown and yellow powdery precipitates.

$$K^+ + 3Fe^{3+} + 2SO_4^{2-} + 6H_2O \longrightarrow KFe_3(SO_4)_2(OH)_6 + 6H^+ \qquad \textbf{Equation 1.16}$$

$$K^+ + 3Fe^{3+} + 2HSO^{4-} + 6H_2O \longrightarrow KFe_3(SO_4)_2(OH)_6 + 8H^+ \qquad \textbf{Equation 1.17}$$

(Brierley & Brierley 1995)

The formation of these precipitates may become excessive and inhibit the recovery of metals by subsequent chemical methods due to the coating of the target metals with the jarosites. Similarly, inhibition of biooxidation may occur by the jarosite coating preventing the adequate attachment of the bacteria to the mineral surfaces (Dew *et al*, 1997). These precipitates also make observation and enumeration of bacteria extremely difficult due to the formation of bacterial-jarosite aggregates.

More recently molecular data and increased culturing success of halotolerant acidophiles have improved and corroborated the belief that these bacteria are more widespread in nature than originally thought. Therefore there have been more frequent reports on the assessment of the growth of such bacteria on pyritic ore (Bond *et al*, 2000; Bond & Banfield, 2001; Holden *et al*, 1999).

During a program to evaluate halotolerant acidophiles for the bioremediation of metal contaminated harbour sediments, the leaching capacity of a halotolerant Sulfobacillus-like isolate (A19-22) was assessed by Holden *et al* (1999). Iron dissolution rates by this isolate were relatively slow in pyrite cultures. After a residence time of 20 days isolate A19-22 had solubilised 22.8% of the total iron at 0.5% NaCl, 16.3% of the total Fe at 1% NaCl and only 8.15% of the total Fe available in the medium at 4% NaCl. The investigators also showed that the total leaching decreased with increasing salt concentration (Holden *et al*, 1999).

Deveci (2002) reported the effect of salinity on the oxidative activity of different acidophilic bacteria during the bioleaching of a sulphidic ore sample. Deveci noted that

a mixed enrichment culture (isolated from Wheal Jane Mine, Cornwall, UK) extracted 30% of the total available iron at 3% (w/v) chloride and 1% (w/v) ore load after 12.5 days. At 1% chloride the iron extraction was only 35% under the same conditions.

Investigation of the occurrence and characteristics of novel salt-tolerant acidophiles that are capable of carrying out bioleaching processes at high salinity would be of great value, both from a microbial ecological and a biotechnological perspective.

1.6 Aims and Objectives of this study

From a review of the literature and the mining industry interest in the potential biotechnological use of salt-tolerant acidophiles, it was found that there was a gap in the knowledge regarding the diversity of acidophilic, iron-oxidising bacteria that also tolerated growth at high salt levels. It was noted that there was a great deal of interest in the occurrence of these bacteria and their potential role in iron and sulphur-cycling in marine areas, and their potential use in biomining processes. However, research has not kept pace with this interest and is lacking in the description of these bacteria and assessment of their physiology under different conditions.

The objectives of this study were to answer and expand on the following research questions:

Can novel, halotolerant, iron-oxidising bacteria be isolated from marine and estuarine areas of known metal contamination?

What are the growth characteristics of these isolated bacteria under different conditions of salinity, pH, temperature and nutrient source?

Can this type of bacteria offer a potential competitive advantage for mining operations via biomining of metals at high salinity?

The aim of this study is to reduce this gap in the knowledge by isolating halotolerant, acidophilic, iron-oxidising bacteria that are capable of growth on pyritic ores. The growth physiology of these isolates under different chemical and physical growth conditions will be characterised. The phylogeny of these bacteria in relation to other characterised acidophiles will be assessed to gain more information regarding the diversity of this type of microorganism. In addition, an important goal is to determine the growth rates and iron dissolution kinetics of these bacteria when grown on metaliferrous ores and this will provide important information on the utility of such microorganisms in elevated salts biomining technology.

Chapter Two

Materials and Methods

Chapter Two: Materials and Methods

2.1 Characterised bacterial strains used in this study

The bacterial strains used in this study are outlined in Table 2.1 below.

Table 2.1 Bacterial strains used in this study

Bacterial strain name	Source	Identification no.	Reference
Thiobacillus prosperus	DSMZ	5130	Huber & Stetter, 1989
Thiobacillus ferrooxidans (reclassified as *Acidithiobacillus ferrooxidans*)	ATCC	23270	Kelly & Wood, 2000
Acidiphilum sp.	NCIMB	11745	Johnson, 1995
Acidiphilum SJH	Kindly donated by Dr. D.B.Johnson, Bangor University		Johnson, 1995
Leptospirillum ferrooxidans	ATCC	53992	Premuzic & Lin, 1994
Alicyclobacillus hesperidum	DSMZ	12489	Albuquerque *et al.* 2000
Alicyclobacillus cycloheptanicus	DSMZ	4006	Deinhard *et al.* 1987
Sulfobacillus acidophilus	DSMZ	10332	Norris *et al*, 1996
Sulfobacillus disulfidooxidans	DSMZ	12064	Dufresne *et al*, 1996

DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - German Collection of Microorganisms and Cell Cultures

http://www.dsmz.de

NCIMB – National Collections of Industrial, Food and Marine Bacteria

http://www.ncimb.co.uk

ATCC - American Type Culture Collection, The Global Bioresource Centre™

http://www.attc.org

2.2 Media and culture conditions

The sources of chemicals used are outlined in appendix D

2.2.1 T. prosperus medium

Modified from medium M477 (Huber & Stetter, 1989)

Contains per litre of distilled water (dH_2O); KCl 0.33g, $MgCl_2.6H_2O$ 2.75g, $MgSO_4.7H_2O$ 3.45g, NH_4Cl 1.25g, $CaCl_2.2H_2O$ 0.14g, K_2HPO_4 0.14g, KH_2PO_4 0.14g, NaCl 0.5g, $NiCl_2.6H_2O$ 2.0 mg, trace element solution (see section 2.2.3 for recipe) 10.0 ml, distilled water to 1 litre. The medium was adjusted to pH 2.5 with H_2SO_4 and then autoclaved. $FeSO_4.7H_2O$ solution (10% from a 20% w/v filter sterilised stock solution pH 2.0) was added aseptically when medium was cool. If ore was used as the substrate this was added before autoclaving. Cultures were grown at 35°C with shaking.

2.2.2 At. ferrooxidans medium

Contains per litre; $(NH_4)_2SO_4$ 3g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.5g, KCl 0.1g, Ca $(NO_3)_2$ 0.01g made up to 1 litre with dH_2O and pH was adjusted to pH 2 with concentrated H_2SO_4, medium was aliquoted and autoclaved. A solution of filter-sterilised ferrous sulphate was then added to the medium to a final concentration of 100mM. Cultures were grown at 28°C with shaking.

2.2.3 Media used for enrichment and growth of the isolated bacteria

Ferrous iron saline medium/FSM (for growth of environmental isolates)

Basal salts solution 100 ml, distilled water 900 ml, yeast extract 0.2 g, trace element solution 0.5ml, sea salts (Sigma) 30g. The pH was adjusted to pH 2.0 with H_2SO_4, and the medium was dispensed into flasks and autoclaved. Sea salts or NaCl was omitted for non-saline medium (FM) and yeast extract was omitted for autotrophic medium. A stock solution of filter-sterilised ferrous sulphate was added after autoclaving to produce a final iron concentration of 100 mM. For heterotrophic medium, the addition of ferrous sulphate was omitted and 0.5 g of yeast extract was added instead of 0.2 g.

Pyrite saline medium/PSM (for growth of environmental isolates)

Basal salts solution 100 ml, distilled water 900 ml, powdered pyrite (FeS_2) 2 g, yeast extract 0.2 g, trace element solution 0.5 ml, sea salts (Sigma) or NaCl 30 g. The pH was adjusted to pH 2.0 with H_2SO_4; the medium was dispensed into flasks and autoclaved. Sea salts or NaCl was omitted for non-saline medium (PM) and yeast extract was omitted for autotrophic medium.

Yeast extract saline medium/YSM (for heterotrophic growth)

Basal salts solution 100 ml, distilled water 900 ml, yeast extract 0.5 g, trace element solution 0.5 ml, sea salts (Sigma) or NaCl 30 g. The pH was adjusted to pH 2.0 with H_2SO_4, the medium was dispensed into flasks and autoclaved. Sea salts or NaCl was omitted for non-saline medium (YM).

Basal salts solution (Johnson, University of Wales at Bangor, 2001 personal communication)

Contains per litre of distilled water; $(NH_4)SO_4$ 1.5g, KCl 0.5g, $MgSO_4.7H_2O$ 5.0g, KH_2PO_4 0.5g, $Ca(NO_3)_2$ 0.1g (pH was adjusted to pH 2.5 with concentrated H_2SO_4).

Trace elements solution

Contains per litre of 0.01M H_2SO_4; $ZnSO_4.7H_2O$ 10g, $CuSO_4.5H_2O$ 1g, $MnSO_4.4H_2O$ 1g, $CoSO_4.7H_2O$ 1g, $Cr_2(SO_4)_3.15H_2O$ 0.5g, $Na_2B_4O_7.10H_2O$ 0.5g, $NaMoO_4.2H_2O$ 0.5g, $NaVO_3$ 0.1g.

2.2.4 Ferrous iron overlay plates (Johnson, University of Wales at Bangor, 2001 personal communication)

Solution A: 40 ml basal salts solution, 0.4 ml trace element solution, 0.1 g tryptone soya, 250 ml distilled water, pH was adjusted to 2.5 with H_2SO_4

Solution B: 2 g agarose, 100 ml distilled water

Solution C: 1M ferrous sulphate, pH 2.0, filter-sterilised

Solutions A and B were autoclaved separately and cooled to 45°C. The two solutions were mixed and 10 ml of solution C added. The combined medium was split into two

sterile bottles 70:30. The greater amount of medium was inoculated with an active culture of *Acidiphilum* SJH and poured immediately into 15 plates. The plates were allowed to gel and the remaining medium poured on top. Plates were stored at 28°C for up to a week to allow the *Acidiphilum* sp. to grow and were then inoculated with bacteria.

2.2.5 Solid ferrous iron medium

Solution 1: agarose 5 g, dH_2O 200 ml
Solution 2: basal salts 100 ml, trace element solution 0.5 ml, yeast extract 0.5 g, sea salts (Sigma) or NaCl depending on salinity of medium, dH_2O 650 ml, pH adjusted to 2.0 with concentration H_2SO_4.
Solution 3: 200 gl^{-1} $FeSO_4.7H_2O$ 50 ml, pH adjusted to pH 2.0 with H_2SO_4 and filter-sterilised.

Solutions 1 & 2 were autoclaved separately and were combined when cooled to 50°C, solution 3 was then added and plates were poured. The plates should not be stacked when pouring as the longer cooling time may cause the agarose to hydrolyse and therefore the plates will not set. Sea salts or NaCl were omitted for non-saline medium.

2.2.6 Estuarine enrichment medium EEM

Contains per litre dH_2O; $NaHCO_3$ 2.5 g, $CaCl_2.2H_2O$ 1.0 g, KCl 0.1 g, NH_4Cl 1.5 g, NaH_2PO_4 0.6 g, NaCl 11.7 g, $MgCl_2.6H_2O$ 5.3 g, $MgSO_4.7H_2O$ 0.1 g, $MnCl_2.4H_2O$ 5 mg, $NaMo_4.2H_2O$ 5 mg, $NaCH_3COO$ 2.7 g. (50 mg of yeast extract was added for an additional carbon source). Various iron sources were added and the pH was adjusted to 2.0 with H_2SO_4, the medium was then dispensed and autoclaved (Lovely, 1992).

2.2.7 Alicyclobacillus hesperdum medium (DSMZ 12489)

DSMZ medium 402: Solution A: $CaCl_2.2H_2O$ 0.25 g, $MgSO_4.7H_2O$ 0.5 g, $(NH_4)_2SO_4$ 0.2 g, yeast extract 2 g, glucose 5 g, K_2HPO_4 3 g, dH_2O for liquid medium 1000 ml, (for solid medium 500 ml), trace element solution 1 ml. The pH was adjusted to 4 by addition of concentrated H_2SO_4. Solution B: agar 15 g, dH_2O 500 ml. These solutions were autoclaved separately. For solid medium solutions A and B were combined. Cultures were grown at 50°C.

2.2.8 Alicyclobacillus cycloheptanicus medium (DSMZ 4006)

DSMZ medium 402 was used for culture of this strain (see section 2.2.7) but 5 gl^{-1} yeast extract was used instead of 2 gl^{-1}. Cultures were grown at 45°C.

2.2.9 Sulfobacillus acidophilus medium (DSMZ 10332)

DSMZ medium 709: per litre of dH_2O; $MgSO_4.7H_2O$ 0.5 g, $(NH_4)_2SO_4$ 0.4g, K_2HPO_4 0.2 g, KCl 0.1 g. The pH was adjusted to 2.0 using concentrated H_2SO_4. For heterotrophic medium a stock solution of filter-sterilised $FeSO_4.7H_2O$ was added to obtain a final concentration of 10 mgl^{-1} and a stock solution of filter-sterilised yeast extract was added to a final concentration of 0.25 gl^{-1} after autoclaving. For autotrophic medium a stock solution of $FeSO_4.7H_2O$ (pH 1.7) was added after autoclaving to obtain a final concentration of 13.9 gl^{-1}. Cultures were grown at 45°C.

2.2.10 Sulfobacillus disulfidooxidans medium (DSMZ 12064)

DSMZ Medium 812: per litre of dH_2O; $(NH_4)_2SO_4$ 3 g, KCl 0.1 g, KH_2PO_4 0.5 g, $MgSO_4.4H_2O$ 0.5 g, $Ca(NO_3)_2.4H_2O$ 0.1 g, yeast extract 0.1 g. The pH was adjusted to pH 2.25 before autoclaving and before use 10 ml of filter sterilised 10% (w/v) glutathione solution was added. Cultures were grown at 35°C.

2.2.11 Acidophilum spp. medium (NCIMB 11745)

NCIMB Medium 155: per litre of dH_2O; $MgSO.7H_2O$ 0.5 g, $(NH_4)_2SO_4$ 0.1 g, KH_2PO_4 50.0 mg, KCl 50.0 mg, $Ca(NO_3)_2$ 10.0 mg, mannitol 1.0 g, tryptone soya broth 0.1 g. The pH was adjusted to 3.5 with concentrated H_2SO_4, and autoclaved. For agar, the medium was prepared at double strength in 500 ml dH_2O without agar, was autoclaved and added to an equal volume of hot sterile, double strength agar solution (12 g agar in 500 ml dH_2O, pH 7.0). Cultures were grown at 28°C with shaking.

2.2.12 Leptospirillum ferrooxidans medium (ATCC 53992)

per litre of dH_2O; $(NH_4)_2SO_4$ 132 mg, $MgCl_2.6H_2O$ 25 mg, $CaCl._2H_2O$ 147 mg, KH_2PO_4 27 mg, 1 ml of trace elements solution (section 2.2.3). The medium was adjusted to pH 1.8 with concentrated H_2SO_4, dispensed and autoclaved. A stock solution of filter-sterilised $FeSO_4.7H_2O$ (pH 1.8) was added prior to use to obtain a final iron concentration of 100 mM.

2.2.13 TSM solid medium (Bianchi *et al*, 1989)

Solution A: $(NH_4)_2SO_4$ 3.0 g, $MgSO_4.7H_2O$ 0.5 g, KCl 0.1 g, K_2HPO_4 0.05 g, $Ca(NO_3)_2.4H_2O$ 0.015 g were dissolved in 600 ml distilled water acidified to pH 2.0 with concentrated H_2SO_4. The solution was autoclaved at 121°C for 15 minutes.

Solution B: $FeSO_4.7H_2O$ 22.0 g in 150 ml dH_2O acidified to pH 2.0 with H_2SO_4. The solution was filter sterilised and then warmed to 60°C in a water bath.

Solution C: agarose 8 g in 250 ml dH_2O at pH 7.0. The solution was autoclaved at 121°C for 15 minutes.

Solutions A, B and C were maintained at 60°C and mixed together prior to pouring.

2.3 Monitoring of Cell Growth

2.3.1 Direct counting of planktonic cells

Cells were enumerated by direct counting using a Neubauer IMPROVED BRIGHT-LINE haemocytometer (depth 0.100 mm, area of squares 0.0025 mm^2) with a Unilux-12 light microscope under a magnification of x1000 (oil immersion lens). The number of cells were counted in 16 random squares. The average number of cells per square was then multiplied by 4×10^6 to give the number of cells ml^{-1} (see equation 2.1 below).

$$\text{Cells ml}^{-1} = \frac{\text{total number of cells in 16 squares}}{16} \times (4 \times 10^6) \qquad \text{equation 2.1}$$

2.3.2 Specific growth rate constant calculation

Specific growth rate constants were then calculated as shown in equation 2.2 below.

$$k = \frac{(\lg N_t - \lg N_0)}{0.301(t_t - t_0)} \qquad \text{equation 2.2}$$

k = specific growth rate constant

$\lg N_t$ = log cells ml^{-1} at time t

$\lg N_o$ = log cells ml^{-1} at time t_0

2.3.3 Use of a fluorescence stain to count cells under UV light

A 1 $mgml^{-1}$ stock solution of fluoroscein diacetate (FDA) was prepared in acetone, this was transferred in 1ml aliquots to microfuge tubes and stored at –20°C. A drop of this stock solution was added to 1 ml of culture and was left for 10 mins for the stain to be taken up. The cells were counted using a Neubauer haemocytometer viewed under ultraviolet light using a Zeiss Epiflurosence Microscope to enumerate viable cells. The light source was switched between visible to UV in order to see the squares on the haemocyctometer. The number of viable cells ml^{-1} was calculated in the same manner outlined in Section 2.3.1.

2.3.4 Use of oxalic acid for release of cells attched to particles or which are enclosed in iron precipiates (modification of method from Ramsay et al, 1988)

Cells were released from jarosite precipitates by adding a 1 ml culture sample to 5 ml of 1% (w/v) oxalic acid. The solution was mixed and the cells were counted immediately before the cells were damaged by the oxalic acid. The oxalic acid dissolves intermediate precipitation products of iron oxidation such as jarosite.

2.3.5 Use of sonic water bath to release cells attached to ore particles

Cells grown on ore samples were difficult to observe under the microscope because many attached to the ore particles therefore a method was developed to release these attaced cells. Samples of ore cultures (1ml) were dispensed into microfuge tubes and put in the sonic water bath for 5 min exactly. This action was found to shake attached cells off the ore particles and allowed the cells to be observed more easily. The cells were observed under a light microscope at a magnification of x1000 (oil immersion lens).

2.3.6 Measurement of growth in heterotrophic bacterial cultures

The growth of heterotrophic cultures was monitored by reading the absorbance of the cultures in a 1 ml cuvette using a Bio-Rad Smart Spec spectrophotometer at wavelength of 600nm. A calibration curve was created by directly counting the cells in a haemocyctometer (see Section 2.3.1) and measuring the absorbance at 600 nm and ploting these values on a graph. This graph was then used to determine the number of cells ml^{-1} in cultures using the absorbance value.

2.4 Determination of ferrous iron and total dissolved iron concentrations in bacterial cultures

Samples from ferrous iron cultures were used directly for iron determination. However, the particles from ore grown cultures studied in the bioleaching experiments interfered with iron determination methods and cell counts and so the following method was used in order to separate the ore particles from the medium;

A sample (1.2 ml) was taken aseptically from the test ore cultures and placed in a 1.5 ml microfuge tube. The tubes were spun for 30 sec at 13,000 rpm to remove any suspended ore material. 0.5 ml of the supernatant was transferred to 0.5 ml 6M HCl in a 1.5 ml microfuge for later analysis of the target metal by atomic absorption spectrophotometry. 0.5 ml of the supernatant was added to a plastic test tube for ferrous iron content determination. 100 µl of the supernatant was added to a plastic test tube for total dissolved iron content determination. 30 µl was used to count total planktonic cell number using a Neubauer haemocytometer.

A modification of the phenanthroline method of iron determination from Standard Methods in Wastewater analysis (Rand *et al*, 1976) was used to quantify Fe^{2+} and Fe^{3+} levels in culture samples using chelation with 1,10-phenanthroline (see below).

2.4.1 Ferrous iron determination using 1, 10-phenanthroline indicator

See Section 2.4.4 for composition of solutions used.

For ferrous iron grown bacterial samples:

20µl of sample was added to 5 ml of 0.5 M HCl in an acid-washed universal bottle, the bottles were then inverted to mix and left for 15 min to bring the iron into solution. Duplicate tubes were set up containing 800 µl of phenanthroline indicator solution, 400 µl ammonium acetate (Section 2.4.4), and 2.3 ml distilled water. After 15 min, 0.5 ml of the sample in HCl was added to each of the duplicate tubes and the absorbance was measured using a Jenway 6405 UV/Vis spectrophotometer at a wavelength of 510nm.

For ore grown bacterial cultures:

500 µl of sample was added to 1 ml of 0.5M HCl. This was inverted to mix and left for 15 minutes to extract. Then the same protocol for ferrous iron determination was then followed.

2.4.2 Total iron determination using 1,10-phenanthroline indicator

For ferrous iron grown bacterial samples:

20 µl of sample was added to 5 ml of hydroxylammonium chloride solution (Section 2.4.4) and this was left to extract for 15min. Duplicate tubes were set up using the same protocol as that for ferrous iron determination. 0.5 ml of sample was added to duplicates and absorbance was read using a spectrophotometer at a wavelength of 510 nm.

For ore grown bacterial cultures:

100µl of sample was added to 1 ml of hydroxylammonium chloride solution (Section 2.4.4) and this was left to extract for 15 min. Duplicate tubes were set up using the same protocol as that for ferrous iron determination (ferrous iron grown cultures). 0.5 ml of sample was added to the duplicate tubes and absorbance was read using a spectrophotometer at a wavelength of 510 nm.

The test tubes used in this method were acid washed using 6 M HCl between assays to remove traces of iron that would interfere with the accuracy of the assay.

2.4.3 Preparation of standard curves for use with the 1,10-phenathroline indicator iron determination method

A stock solution of $FeSO_4.7H_2O$ was prepared at a concentration of 100 mM by adding 2.78 g $FeSO_4.7H_2O$ to 100 ml water (which had been acidified to pH 2.5 with a few drops of concentrated H_2SO_4). Dilutions ranging from 1 mM – 100 mM were prepared using this stock solution.

The diluted samples were used as detailed in sections 2.4.1 and 2.4.2. Four separate calibration curves were constructed for ferrous iron and total iron (using both ferrous and ore culture methods).

New calibration curves were constructed when new reagents were made up, in order to maintain the accuracy of the assay. The obtained gradients were then used to determine iron concentrations.

(See Appendices Ci and Cii for example calibration curves for ferrous and total iron concentrations determined using the pyrite method).

2.4.4 Solutions used in iron determination:

Phenanthroline indicator solution: 100 mg of 1,10-phenanthroline hydrate was dissolved in <80 ml dH_2O. Two drops of concentration HCl was added to aid dissolution and the solution was made up to 100 ml.

Hydroxylammonium chloride reagent: 1.25 g $HONH_3Cl$ was dissolved in 600 ml water and 12 ml concentrated HCl was added with mixing, in a fume cupboard.

Ammonium acetate solution: 62.5 g $CH_3CO_2NH_4$ was added to 37.5 ml dH_2O and 175 ml glacial acetic acid was added in fume cupboard.

0.5 M HCl: 44.5 ml of concentrated HCl was added to 955.5 ml dH_2O in a fume cupboard with mixing.

2.5 DNA extraction from bacterial ore cultures and PCR amplification

2.5.1 Preparation of bacterial pellets from ore cultures

Two 50 ml cultures were spun down in a centrifuge at 4000 rpm for 10 min. The supernatant was discarded and the pellets were then transferred into microfuge tubes. The tubes were placed in a sonic water bath for 5 min to detach as many of the attached bacteria from the ore particles as possible. The tubes were then centrifuged at 5000 rpm for 30 sec to separate the ore from the medium. The supernatant from both tubes was combined and centrifuged for 10 min at 13,000 rpm. The ore particles remaining were resuspended in PBS and the process repeated to retrieve more of the attached bacteria in the pellet. This pellet was then combined with the previously obtained bacterial pellet.

2.5.2 Extraction and precipitation of DNA

The DNA was then extracted from the cell pellet using a Qiagen DNeasy Tissue Kit. The obtained DNA was then subjected to ethanol precipitation to purify and concentrate the DNA, as follows: 1/10 volume of 3 M sodium acetate (pH 5.2), and then two volumes of the new total volume of cold 96% ethanol was added and the tube vortexed briefly. The sample was then precipitated for 20 min at –70 °C or overnight at –20°C. The tube was then centrifuged for 15 min at 13,000 rpm, the supernatant was removed and the pellet was washed with 250 µl of 70% ice cold ethanol. The tube was then centrifuged for 5 min at 13, 000 rpm in the same orientation, the supernatant removed and remaining ethanol was evaporated on a heating block at 80°C. The DNA pellet was finally resuspended in 50µl of TE buffer.

2.5.3 PCR reagents and conditions

PCR tubes were set up as follows for a 100 µl reaction:

7 µl 10X NH_4 buffer
3 µl 50mM $MgCl_2$
10 µl 2 mM dNTP mix
0.5 µl forward primer (concentration of primers was 100 pmol/ml)
0.5 µl reverse primer
1 µl DNA sample
0.5 µl Taq polymerase (0.5 unit)
Millipure water up to a total of 100 µl

The PCR was then run on a Helena Biosciences Proteus Thermocycler machine under the following conditions:

Temperature	Time of hold	Number of cycles
92°C	2 min	1
94°C	30 sec	
40°C	1 min	5
94°C	30 sec	
50°C	1 min	
72°C	3 min	30

The obtained PCR products were electrophoresed, alongside a 100bp DNA ladder on a 1% agarose gel (0.3 g agarose in 30 ml 1X TAE buffer) in a LifeTechnologies GibcoBRL Horizon® 58, Horizontal Gel electrophoresis tank. 1 X TAE was used as the running buffer. The gel was prestained before running, with 0.75µl of 1 $mgml^{-1}$ ethidium bromide solution. The gel was run for approximately 1 h at 70V and the bands were then viewed using a UVP Transilluminator UV light box. A picture was taken of

the obtained bands using a UVP monochrome CCD camera apparatus with gel documentation system and video graphic printer, and the gel disposed of.

The PCR products were then sequenced as detailed in section 2.6 and the obtained sequences were run through a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6 Preparation of PCR products for DNA sequencing

2.6.1 Purification of PCR products for sequencing

PCR products were purified using a PCR purification kit CONCERT™ Rapid PCR Purification System to get rid of unbound primer that would interfere with subsequent sequencing reactions.

An aliquot of TE buffer was pre-warmed to 65-70°C. 400 µl of Binding Solution (H1) was added to the amplification reaction and this was mixed thoroughly. A cartridge was placed into a 2 ml wash tube and the sample was loaded into the cartridge. This was centrifuged at 13,000 rpm for 1 min and the flow-through was discarded. The cartridge was placed back into the wash tube and 700 µl of Wash Buffer (H2, containing ethanol) was added. The tube was centrifuged at 13,000 rpm for 1 min and the flow-through discarded. The tube and cartridge was centrifuged again at 13,000 rpm for 1 min to remove all residual wash buffer. The cartridge was placed in a 1.5 ml recovery tube and the DNA was eluted by adding 50 µl of the pre-warmed TE buffer directly to the centre of the cartridge. This was incubated at room temperature for 1 min and then centrifuged at 13,000 rpm for 2 min.

Purified PCR products were then used in the following sequencing reactions

***2.6.2 Cycle sequencing reaction*:** using BigDye 1.0 from ABI PRISM DNA sequencing kit

Big Dye 1.0 Terminator Cycle sequencing ready reaction mix, 2.0 µl, 2.5 x sequencing buffer 2.0 µl, template (PCR product 2-100 ng, usually around 1.0 µl), primer 3.2 pmol, (usually around 0.5 µl) and millipure water as required to a total volume of 10µl.
The 5X sequencing buffer contained: 400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0

The reagents were mixed well and spun briefly and then subjected to the sequencing cycle (2.6.3).

2.6.3 Cycle sequencing reaction conditions

Thermal cycle was 5 min at 98°C, followed by 25 cycles of:

96°C for 10 sec

50°C for 5 sec

60°C for 4 min

and the samples were held at 4°C until purification.

2.6.4 Purification of sequencing reaction

The contents were then spun down and purified as follows: the product was transferred to a 1.5 ml microfuge tube and 2 µl of 3M sodium acetate were added. 50 µl of ice cold 100% or 95% ethanol was then added and the contents were vortexed briefly. This mixture was left for 15 min at room temperature to precipitate the products. The tube was then centrifuged at 13,000 rpm for 20 min and the orientation of the tubes was noted. The ethanol was removed and the samples washed with 250 µl of 70% ethanol and the samples were centrifuged for a further 5 min in the same orientation. The ethanol was then removed and the samples dried at 90°C on a heating block.

2.6.5 Denaturation of sequencing reactions

25µl of TSR buffer was added to the dried DNA. The samples were vortexed briefly and centrifuged. They were then heated for 2 min at 90°C, vortexed and spun briefly and transferred to sequencing tubes. The samples were chilled on ice or frozen until being loaded onto the sequencer.

2.6.6 Sequence determination

The samples were analysed on an ABI PRISM™ 310 Genetic Analyser (Applied Biosystems), together with Autoassembler™ Software (Version 2.1) and GeneScan® Analysis Software (Version 3.4.1). The output obtained was in the form of a chromatogram and this was viewed using Chromas software package. The obtained sequences were checked and cleaned-up using the peak pattern observed on the corresponding chromatogram.

2.7 Phylogenetic Characterisation of Environmental Isolates

2.7.1 Determination of the sequence of 16S rDNA

DNA prepared from the isolates was used to determine the sequence of the 16S rDNA gene. PCR products were obtained using the PCR protocol outlined in section 2.5.3 using the primers 27F and 1541R. These products were purified using the CONCERT Rapid PCR purification system (section 2.6.1) and then subject to a sequencing reaction described in section 2.6.2 using the primers detailed in Table 2.2 below.

Table 2.2 16S rRNA DNA primers

Primer ID	Sequence	Tm (°C)
9F	5'-GAGTTTGATCCTGGCTCAG-3'	56.7
27F	5'-AGAGTTTGATCMTGGCTCAG-3'	56
342F	5'-CTGCTGCSYCCCGTAG-3'	58
515F	5'-GTGCCAGCAGCCGCGGT-3'	62.4
685F3	5'-GTAGCGGTGAAATGCGTAGA-3'	50
785F	5'-GGATTAGATACCCTGGTAGTC-3'	57.9
1099F	5'-GCAACGAGCGCAACCC-3'	56.9
534R	5'-GTATTACCGCGGCTGCTG-3'	58.2
685R3	5'-TCTRCGCATTYCACCGCTAC-3'	60
802R	5'-TACCAGGGTATCTAATCC-3'	51.4
907R	5'-CCGTCAATTCMTTTRAGTTT-3'	54
1115R	5'-AGGGTTGCGCTCGTTG-3'	54.3
1492R	5'-TACGGYTACCTTGTTACGACTT-3'	57.5
1541R	5'-AAGGAGGTGATCCAGCC-3'	55.2

2.7.2 Assembly of contiguous 16S rRNA DNA sequences

The sequences obtained were then cleaned up using the peak patterns on the chromatogram. These cleaned-up sequences were then put together to form contiguous 16S rRNA DNA sequences by using the CAP EST ASSEMBLER (FIRC Institute of Molecular Oncology, Italy – http://bio.ifom-firc.it/ASSEMBLY/assemble.html) and searches for similar sequences were performed using NCMB BLAST software http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al*, 1997) and bacteria with high identities were noted, and a similarity matrix was constructed for the isolated bacteria and their closest relatives using the sequences identity values obtained. Clustal W (CMBI, Centre for Molecular and

Biomolecular Informatics-http://www.cmbi.kun.nl/) was then used to align the obtained 16S rDNA sequences with those of characterised related bacteria and calculate nucleotide substitution rates and this alignment data in PHYLIP format was then fed into TREEVIEW (Page, 1996) and phylogenetic trees were constructed.

2.8 Environmental Sampling to isolate salt tolerant iron-oxidising bacteria

2.8.1 Sampling in East Fife, Scotland, UK

Samples were taken from marine, estuarine and coastal areas of Fife, Scotland UK. The areas chosen were areas known to have a high concentration of metals or on sites of abandoned mine discharge. These sites are outlined in Table 2.3.

Table 2.3 Description of East Fife, Scotland sample sites, and material sampled

SAMPLE SITE AND GRID REFERENCE	DESCRIPTION OF SITE	MATERIAL SAMPLED
Inverkeithing NT134819	disused boat yard opposite quarry site- rusting metal submerged in water	sediment, water and rusty metal, pH 5.0
Burntisland NT264862	rusty metal pipes, submerged on beach and tidal	water and rusty metal, pH 6.0
Aberdour NT407034	orange coloured iron oxide discharge from stream running onto beach	seawater and discoloured sand, pH 5.0
East Wemyss NT343968	beach polluted with oil	sediment samples, pH 7.0
St. Andrews NO504184	recreational beach, open sea	water samples and submerged sand, pH 7.0
Anstruther NO576036	harbour	sediment, pH 6.0

2.8.2 Sampling in Cornwall, England, UK

A programme of sampling from coastal and estuarine areas was undertaken in Cornwall, England with a view to the isolation of halotolerant iron and sulphur-oxidising bacteria. The sites included areas of known acid mine drainage contamination, mine tailing pollution and seawater exposed pyritic rock surfaces. Over 100 samples were collected from 10 different sites (the sites are outlined in Table 2.4). All the samples were logged

and allocated codes for subsequent culture identification (location maps are shown in Appendices A.i-A.xi. The salinity and pH of the samples were measured to monitor the immediate sampling environment. Sediment and water samples were taken from sites taking care to use sterile equipment and sample bottles to minimise outside contamination of the sample material. Core samples were taken using bore apparatus with bungs on either end. Spatulas were sterilised between sites by surface sterilising with ethanol.

Table 2.4 Location and description of Cornwall samples

SAMPLE SITE AND GRID REFERENCE	DESCRIPTION OF SITE	MATERIAL SAMPLED
Calenick Creek SW827432	Tidal estuary with mine tailing pollution in sediments	Mine tailing sediment with ferric oxide veins
Cligga Head SW737537	Mineralised greisen containing pyrite, abandoned tin mine – network of caves which are tidal	Scrapings from pools in caves (abandoned tin mine) with iron oxide staining, water samples from tidal caves, sediment samples from the base of pyritic cliffs
Devoran SW797387	Shore near the mouth of Restronguet Creek which has iron oxide stained sediments and is exposed to seawater tidally	Sediment samples and sea water samples from around the iron oxide stained stones on shore
The Gannel SW801609	Tidal sediments high in galena, spalerite – high metals content originates from mine waste following mine closure	Sediment samples with iron oxide veins and pockets, samples with high tailings content

Hayle Estuary SW555375	Inlet with sediments high in mine tailings, - effective sediment trap for mine waste released into the river catchments draining into the estuary	Sediment samples with iron oxide staining in pockets, mine tailings sediment and samples of seawater from the iron oxide areas
Restronguet Creek SW804385	Tidal estuary with Carnon river running through which has brought mine tailings and discharge from the Wheal Jane mine which was closed in 1992 after the mine effluent was discharged into the river and estuary	Samples from the isolated saltwater pools around the Carnon River, which were a green colour, which is indicative of copper pollution. Samples of sediment from the Carnon river and mine tailings from the estuary above
St. Michael's Mount SW514299	Island off Cornwall coast with an abandoned mine that was built into the base of the island. The mine still has an outflow to the South of the Mount	Scrapings from the tidal rocks that were in contact with outflow from an abandoned mine below the Mount. Seawater sample from rock pools with iron oxide staining
Tintagel Head SW053893	Disseminated pyrite in devonian sediments which outcrops onto the beach	Scrapings from the pyritic cliffs in contact with seawater
Tresillion SW860455	Estuary which is highly contaminated with mine tailings from mines in the catchment area above	Sediment samples with iron oxide veins
Trevone (Padstow) SW893764	Pyrite inter-bedded within Firebeacon shales which outcrop onto the beach	Scrapings from the pyritic rocks and cliffs which were tidally in contact with seawater

2.8.3 Sample processing

The samples were initially inoculated into 10ml of ferrous medium (FM), pyrite medium (PM), chalcopyrite medium (PM with chalcopyrite in place of pyrite) containing 30 gl^{-1} sea salts and at a pH value of 2.0 (see Section 2.2.3 for media composition). The enrichment cultures were incubated at 28 °C and 37 °C in stationary flask cultures. They were shaken once a day to increase oxygen availability. Cultures were checked after two weeks of enrichment for signs of autotrophic growth such as visual signs of iron oxidation, formation of iron oxide, and decrease in pH), and those cultures with signs of acidophilic growth were checked under the microscope. Samples were then sub-cultured into the respective medium.

Environmental samples (5 ml liquid sample or 5 g solid sample) were added to 10 ml of saline solution (NaCl 0.25% w/v) and placed in a sonic water bath for 5 minutes as this had previously been found to shake bacteria from sediment or particle surfaces. 100 µl of each sample was then added to separate wells in 96-well microtitre plates containing 100µl of the different combinations of medium shown in Table 2.5.

Table 2.5 Different compositions of media used for bacterial isolation

MEDIUM	SALINITY (gl^{-1})	pH
FM	30	2, 5.5
FM	20	2, 5.5
FM	10	2, 5.5
FM	0	2, 5.5
PM	30	2, 5.5
PM	20	2, 5.5
PM	10	2, 5.5
PM	0	2, 5.5
TM	30	2, 5.5
TM	20	2, 5.5
TM	10	2, 5.5
TM	0	2, 5.5

FM - ferrous iron medium, PM – pyrite medium, TM – same as FM but with tetrathionate (100mM potassium tetrathionate) instead of ferrous iron. (Media composition outlined in section 2.2.3).

Fresh medium was added each week to compensate for evaporation.

2.8.4 Enrichment of bacteria from environmental samples

The media that had been originally inoculated with environmental samples were incubated at 28°C and 37°C for 1 month initially. The iron-oxidising, acidophilic bacteria were enriched by sub-culturing into the corresponding medium every 2 weeks thereafter.

2.8.5 Solid isolation media

Nylon Membrane Culture (L Yan, Heriot-Watt University, UK, personal communication, 2000)

A culture method using nylon membrane as the solid growth strata over a liquid medium was modified for use as an isolation medium for chemoautotrophic bacteria. This method was attempted to overcome the solid growth difficulties of iron and sulphur oxidising bacteria due to the inhibition by available polysaccharides present in traditional heterotrophic medium.

A 5 ml aliquot of sterile ferrous iron medium (FM, section 2.2.3) with 30 gl^{-1} sea salt was dispensed into a sterile shallow dish (top of a 50 ml centrifuge tube) within a small petri dish (Sterilin, petri dishes, diameter 60 mm). A sterile nylon membrane (47 mm diameter Whatman Nylon membrane 0.2 μm filters) was floated on top of the medium (Figure 2.1). Samples were spread on the membrane and the surface was kept moist by the addition of medium every 10 days.

Colonies were observed closely under a dissecting microscope and samples of these were observed using a light microscope to investigate cell morphology. The colonies were picked and used to inoculate different liquid medium.

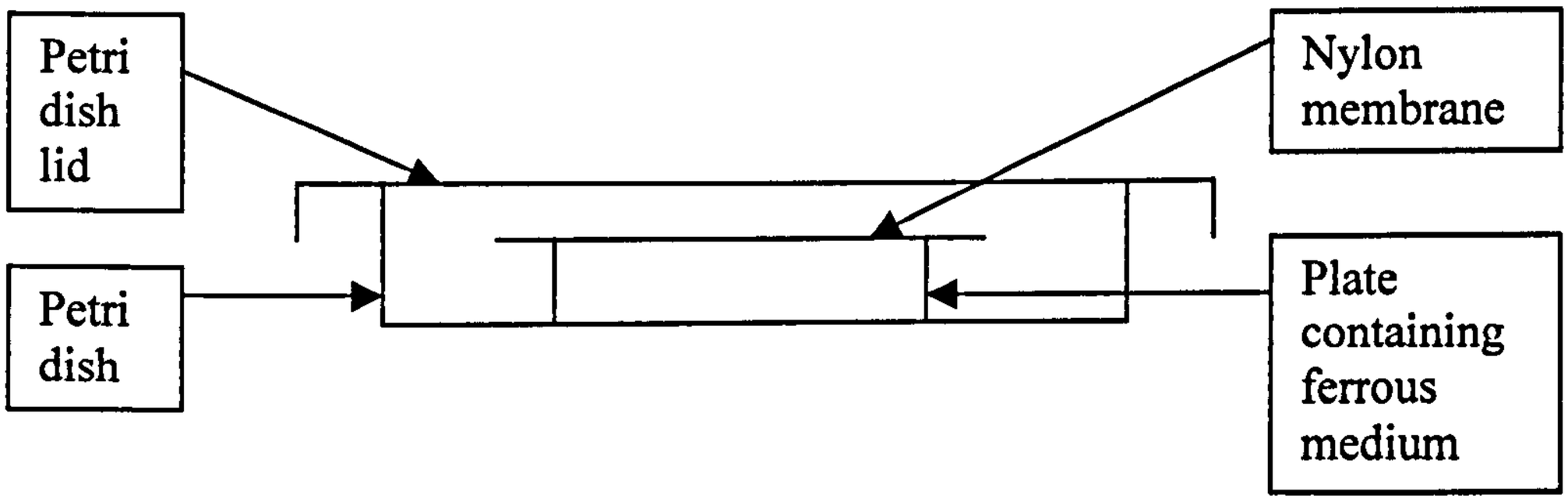


Figure 2.1 Schematic showing the side view of the nylon membrane solid culture apparatus

Overlay agarose plates

Environmental samples which had been shaken in a sonic water bath were streak and spread plated on overlay plates (2.2.4) which had already been incubated at 28°C to allow growth of the *Acidiphilum* SJH strain. The plates were then incubated at 28°C and 37°C.

2.9 Morphology and growth physiology of environmental isolates

Pure colonies were obtained from environmental enrichment cultures (section 2.8) and the following procedures were followed to assess the morphology and growth physiology of the bacteria that were isolated.

2.9.1 Gram stain

Isolates were gram stained and observed under X1000 magnification (oil immersion lens) to assess whether they were Gram-negative or Gram-positive as follows:
A bacterial colony was suspended in a drop of water on a microscope slide and dried above a bunsen flame. The slide was then passed quickly through the flame to fix the cells to the slide. The cooled slide was then stained with crystal violet for 1- 2 min, the stain was then poured off and then stained with Gram's iodine for 1-2 min. After pouring off the iodine, the slide was decolourised by washing briefly with acetone (2-3 seconds). The slide was then washed thoroughly with water to remove the acetone and then flooded with safranin counterstain for 2 min. The slide was washed with water and the excess blotted and the slide dried. Gram-positive bacteria retain the crystal violet stain and are deep purple and Gram-negative bacteria are decolourised can be recognised as lighter pink cells.

2.9.2 Light microscopy

The bacterial isolates were observed using a Zeiss Axiophot Microscope, (Carl Zeiss) under a magnification of X1000 (oil immersion). Average sizes of the bacteria were determined. Photomicrographs were taken of the cells using a JVC KY-F55B colour video camera (Carl Zeiss Ltd, Wewyn Garden City, UK). The software used was a KS300 Imaging System (Imaging Associates Ltd, Thames, UK).

2.9.3 Electron Microscopy

Isolated bacteria were observed by electron microscopy using a Philips XL30 Environmental Scanning Electron Microscope (ESEM) at different stages of growth; during and following sporulation, during growth on different types of media and on different ore substrates. However, technical difficulties were encountered during the use of the ESEM and only one useable image was produced.

2.9.4 Sporulation

The shape of sporulating cells and of the spores produced were observed using a light microscope and electron microscopy. To prove that the isolates sporulate; 10 ml pyrite (with yeast extract and 30 gl^{-1} sea salts) cultures were placed in 85°C water bath for 15 min to kill the viable cells. These cells were then inoculated into pyrite broth and onto solid ferrous iron plates (with yeast extract and 30 gl^{-1} sea salt). The pyrite cultures were checked for growth after 7 days and the plated cultures were checked after 3 days incubation at 37°C. Observation of growth after this treatment suggests spore formation.

2.9.5 Growth physiology

Optimum growth conditions were evaluated by monitoring cell growth and/or iron oxidation under different conditions (medium composition detailed in section 2.2.3)

Temperature

Pyrite medium with yeast extract (0.2 gl^{-1}) and sea salts (30 gl^{-1}) was used (PSM). Duplicate flasks were inoculated with a 1% (v/v) pyrite culture and these were incubated at 28°C, 37°C, 40°C and 50°C on an orbital shaker with a shaking speed of 180 rpm. Cell numbers were monitored every 24 h by direct counting using a haemocytometer.

pH

Pyrite medium with yeast extract (0.2 gl^{-1}) and sea salts (30 gl^{-1}) at pH values of 1.0, 1.5 and 2.0 was used. Duplicate flasks were inoculated with a 1% (v/v) pyrite culture and these were incubated at 37 °C on an orbital shaker with a shaking speed of 180 rpm. Cell numbers were monitored every 24 h by direct counting using a haemocytometer.

Salinity

Pyrite medium with yeast extract (0.2 gl^{-1}) and different amounts of sea salts (0 gl^{-1}, 10 gl^{-1}, 20 gl^{-1}, 30 gl^{-1} sea salts) was used. Duplicate flasks were inoculated with a 1% (v/v) pyrite culture and these were incubated at 37 °C on an orbital shaker with a shaking speed of 180 rpm. Cell numbers (determined by direct counting using a haemocytometer) and the ferrous and total iron (determined using phenanthroline indicator) in solution were measured every 24 h.

Different substrates

Growth was tested on ferrous medium (FM), yeast extract, 30 gl^{-1} sea salts and either 100 mM tetrathionate or 5 gl^{-1} elemental powdered sulphur was added instead of the ferrous iron. Growth was checked microscopically.

Mixotrophic and Heterotrophic growth

Pyrite medium was used with sea salts (30 gl^{-1}) and 0.2 g yeast extract (PSM) and yeast extract medium with sea salts (30 gl^{-1}) and 0.5 g yeast extract (YSM). Duplicate flasks were inoculated with a 1% (w/v) pyrite or yeast extract culture and these were incubated at 37°C on an orbital shaker with a shaking speed of 180 rpm. Cell numbers were measured every 24 h by direct counting using a haemocytometer.

Growth on solid agarose plates

Solid agarose plates were made up as described in Section 2.2.4, with and without sea salts, and with and without yeast extract. The plates were inoculated with the environmental strains grown on different types of media, both by streak plating and spread plating and incubated at 37 °C for approximately 5 days.

2.10 Composition of Rio Tinto ore samples

The following ore samples were provided from various Rio Tinto operations around the world. Rio Tinto technical services personnel performed ore milling/crushing and chemical assays in Rio Tinto's analytical laboratories in Clevedon, UK.

2.10.1 Las Cruces Spanish chalcopyrite

Las Cruces chalcopyrite comes from a copper prospect in Spain and is composed predominantly of pyrite, with copper mineralisation as chalcopyrite. The sample was wet rod milled to provide a d_{80} size of 100 μm (this means that at least 80% of the ore particles are 100 μm or less in diameter). The chemical composition of this ore sample is shown in Table 2.6.

Table 2.6 Chemical assay for Spanish Chalcopyrite ore

Chemical	Concentration (mgg^{-1})
Au	6.01×10^{-7}
Ag	0.0258
Pb	9.854
Zn	9.611
Ni	0.157
Co	0.511
Cd	0.057
Bi	0.689
As	4.506
Sb	0.831
Mn	0.165
Ba	0.186
Cr	0.293
Al	0.021
Mg	negligible
Ca	0.1
Na	0.1
K	0.7
Ti	0.2
Cu	38.4
Fe	420.8
S	459.6
SiO_2	781.4
C (organic)	0.5
Cl	negligible
P	0.220
Hg	0.000041

2.10.2 Lihir Gold Ore

This gold ore sample was from a pressure leach operation on Lihir Island, Papua New Guinea. The principal sulphide mineralisation is pyrite with minor arsenopyrite. The gold principally occurs within the pyrite. Gangue minerals include kaolinite, alunite, sericite, anhydrite, carbonate and quartz. The sample was dried, crushed to pass 2 mm then dry milled in a rod mill. The estimated d_{80} passing size was 125 μm. The chemical composition of this sample is shown in Table 2.7

Table 2.7 Chemical assay of Lihir gold ore sample

Chemical	Concentration (mgg^{-1})
Au	6.354×10^{-6}
Ag	0.0014
Pb	0.087
Zn	0.254
Ni	0.084
Co	0.037
Cd	0.019
Bi	negligible
As	1.617
Sb	0.012
Mn	1.208
Ba	0.374
Cr	0.121
Al	84.8
Mg	5.9
Ca	7.1
Na	3.0
K	61.3
Ti	5.5
Cu	0.3
Fe	77.6
S	72.6
SiO_2	567.5
C (organic)	0.6
Cl	negligible
P	2.091
Hg	0.0005

See appendix Bi for a detailed report of the mineralogy of this ore

2.10.3 Freeport Copper Ore Rough Feed

This copper ore originated from Freeport's Grasberg operation in Irian Jaya, Indonesia. The principal copper mineralisation is chalcopyrite with minor bornite and digenite. Samples were dried and blended, and estimated product d_{80} sizes were 42μm for the concentrate sample and 170 μm for the rougher feed. The chemical composition of this ore sample is shown in Table 2.8.

Table 2.8 Chemical assay for Freeport Rough Feed Ore Sample

Chemical	Concentration (mgg^{-1})
Au	1.067×10^{-6}
Ag	0.0051
Pb	0.052
Zn	0.241
Ni	0.011
Co	0.021
Cd	0.001
Bi	0.006
As	0.011
Sb	negligible
Mn	0.257
Ba	0.475
Cr	0.085
Al	73.8
Mg	11.8
Ca	9.4
Na	16.8
K	52.8
Ti	2.9
Cu	28
Fe	66.5
S	23.6
SiO_2	617.0
C (organic)	0.4
Cl	negligible
P	1.263
Hg	0.000028

2.10.4 Freeport Final Concentrate Sample

The copper ore is the final concentrate from Freeport's Grasberg operation in Irian Jaya, Indonesia. This sample was concentrated by flotation of the Freeport Rough Feed. The principal copper mineralisation is chalcopyrite with minor bornite and digenite. Samples were dried and blended, and estimated product d_{80} sizes were 42μm for the concentrate sample and 170 μm for the rougher feed. The chemical composition of this ore sample is shown in Table 2.9.

Table 2.9 Chemical assay for Freeport Final Concentrate Ore Sample

Chemical	Concentration (mgg^{-1})
Au	1×10^{-5}
Ag	0.0525
Pb	0.246
Zn	2.878
Ni	0.010
Co	0.072
Cd	0.017
Bi	negligible
As	0.023
Sb	negligible
Mn	0.056
Ba	0.016
Cr	0.020
Al	4.9
Mg	0.8
Ca	1.5
Na	1.0
K	3.5
Ti	0.4
Cu	357.7
Fe	255.4
S	300
SiO_2	32.7
C (organic)	2.0
Cl	negligible
P	negligible
Hg	0.00026

2.10.5 Somincor Copper Concentrate Ore Sample

This copper concentrate sample originated from Somincor's Neves Corvo operation in Portugal. Principal sulphide mineralisation is chalcopyrite with gangue pyrite. Minor copper is associated with tetrahedrite, other fahlore minerals and stannite. The sample was dried, blended and the estimated d_{80} product size was 15 μm. Table 2.10 shows the chemical composition of the Somincor ore sample.

Table 2.10 Chemical assay for Somincor Copper Concentrate Ore Sample

Chemical	Concentration (mgg^{-1})
Au	1.59 x 10-7
Ag	0.0702
Pb	4.328
Zn	20
Ni	0.033
Co	0.383
Cd	0.088
Bi	negligible
As	2.648
Sb	0.574
Mn	0.058
Ba	negligible
Cr	0.044
Al	2.7
Mg	0.4
Ca	0.8
Na	0.2
K	1.1
Ti	0.2
Cu	237.6
Fe	308.9
S	300
SiO_2	10
C (organic)	6.7
Cl	negligible
P	negligible
Hg	0.020

2.10.6 Escondida Norte Ore

This ore originates from a copper operation in Chile. It is a low-grade sulphide ore; the sample originates from a porphyry deposit so gangue minerals are probably quartz and altered feldspars and copper is chalcopyrite. The sample was crushed and dry rod milled to provide a product d_{80} size of ~160 μm. Table 2.11 shows the chemical composition of the Escondida ore sample.

Table 2.11 Chemical assay of Escondida ore sample

Chemical	Concentration (mgg^{-1})
Au	6.3×10^{-8}
Ag	0.0025
Pb	0.087
Zn	0.072
Ni	0.099
Co	0.007
Cd	negligible
Bi	negligible
As	0.068
Sb	negligible
Mn	0.110
Ba	0.503
Cr	0.203
Al	73.7
Mg	2.3
Ca	0.8
Na	4.8
K	23.5
Ti	1.6
Cu	19.1
Fe	22.3
S	22.3
SiO_2	781.4
C (organic)	0.5
Cl	negligible
P	0.263
Hg	0.000041

See appendix Bii for a detailed report on the mineralogy of this sample

2.11 Assessment of the biooxidation potential of the environmental isolates

2.11.1 Medium and culture conditions

The ore bioleaching experiments were carried out in pyrite saline medium (PSM) with the ore samples instead of pyrite as the substrate (as described in Section 2.2.3). 2 gl^{-1} yeast extract and 30 gl^{-1} sea salts were added to this medium for assessment of the growth and biooxidation capacity of environmental isolates (designated identification codes - 4G, 5C and Cligga) at high salinity.

The bioleaching potential of these isolates was assessed in shake flasks at 37°C and a shaking speed of 180 rpm. *At. ferrooxidans*, having already been characterised as a bioleaching microorganism, was used as a benchmark bacterial strain and was grown on pyrite saline medium (PSM) with the ore samples instead of pyrite as the substrate and without yeast extract and salt and at 28 °C with shaking at 180 rpm. The initial pH of the medium was always pH 2.0.

Ore material (2 g) was added to 100 ml of pyrite medium (PM with ore sample instead of pyrite, section 2.2.3) in each flask before autoclaving (2 % ore load). The flasks were set up in duplicate in order to calculate standard error values and controls were set up for each ore and were incubated under the same conditions as the bacterial cultures.

The flasks were inoculated with a 10 % (v/v) culture in exponential phase that had been grown up on the same ore for at least 2 successive sub-cultures. The controls were inoculated with the same volume of sterile medium. The ore samples assessed were; Spanish chalcopyrite, Escondida ore, Lihir gold ore, Somincor ore, Freeport rough ore and final concentrate as described in Section 2.10

2.11.2 Measurements taken during biooxidation experiments

Samples were analysed every day for the first 10 days then every two days, up to day 20 and then every 5 days until day 30. Samples were taken as detailed in Section 2.4.

Each sample was analysed for ferrous iron concentration (see section 2.4.1 for methods), total dissolved iron (see Section 2.4.2) using duplicate tubes for each flask sample, and planktonic cell numbers were also measured (see Section 2.3.1). The initial and final pH values were measured on days 0 and 30. The oxidation/reduction potential (or redox potential) was measured on days 0 and 30, using an oxidation/reduction potential (ORP) meter (pocket sized redox meter, Hanna instruments).

Graphs were constructed using the analysed obtained values and the growth rates, rate of iron dissolution, iron oxidation kinetics, rate of dissolution of target metals, change in pH and ORP were determined and compared between isolated strains and with those of *At. ferrooxidans*. Samples were also examined by electron microscopy to compare the appearance of the ore before the bacteria were in contact and following bacterial contact, to observe and any corrosion of the particle surfaces that may have taken place.

Chapter Three

Isolation and characterisation of iron-oxidising bacteria that are both halotolerant and acidophilic

Chapter 3

3.1 Introduction

Isolation of chemolithotrophic bacteria that are both halotolerant and acidophilic has not been widely reported in the literature, despite widespread interest in their potential role in biodegradation of marine iron containing structures, in biogeochemical cycling of iron in high saline areas and their potential biotechnological uses. It is hoped that isolation of this type of bacteria and elucidation of their physiological responses during growth on different culture media and culture conditions will shed light on the microbial ecology of the extreme environments under which these elusive bacterial species survive and thrive.

Such bacteria have been noted as occurring in hydrothermal areas of the oceans where high sulphate and iron levels are found but the extent to which these bacteria tolerate these salts has not been reported (Eberhard, 1995; Harmsen *et al*, 1997; Gugliandolo & Maugeri, 1993). Iron-oxidising bacteria have also been implicated in the biocatalysis of deterioration processes of iron containing structures in marine areas (Herdendorf, 1995). However, research has not kept pace with the interest in these bacteria, as the culprits of biodegradation processes, as agents of biogeochemical cycling and as potential candidates for high salinity bioleaching of metaliferrous ores.

The lack of data in this subject area is, in no small part, due to the difficulty of culture and maintenance of this group of bacteria. The growth of these bacteria is usually inhibited by high concentrations of complex polysaccharides and therefore culture on the commonly used solid bacteriological agar is difficult. Growth is also slower in chemolithotrophic bacteria and final cell concentrations obtained in batch culture are usually considerably less than that of heterotrophic bacteria. This is due to the smaller amount of energy released by using inorganic ions as electron donors, compared with the energy produced from oxidation of organic material observed in heterotrophic bacteria.

Moreover, some studies that have successfully isolated this type of bacteria have reported difficulty in maintaining the viability of their isolates (K. Kamimura, Okayama

University, Japan, personal communication 2002 and D. Barr, RioTinto Plc, Melbourne, Australia, personal communication, 2000).

The complexity of the metabolic processes exhibited by these bacteria as a response to not just one extreme but three extremes (high salinity, low pH and growth on high concentrations of metals that may otherwise be toxic) all conspire to make *in vitro* growth a difficult and slow process. As a result, these bacteria tend to have very long generation times which are characteristic of chemoautotrophic bacteria that must fix carbon dioxide as their carbon source and must oxidise large amounts of inorganic compounds to generate sufficient energy for growth. Mean generation times range from 10 hours to several days (this study).

These factors together have made it an extremely challenging task to determine optimum growth conditions, biooxidation potential and to also ensure the longevity of culture stocks. This chapter describes the enrichment, isolation and characteristics of halotolerant, iron-oxidising bacteria and details optimum growth conditions with regard to temperature, pH, salinity and energy source.

3.2 Enrichment of iron-oxidising, acidophilic, halotolerant bacteria

3.2.1 Introduction

Many of the reports of isolation of halotolerant iron-oxidising bacteria used liquid medium to enrich for these bacteria. Holden *et al* (1999) used a modified artificial seawater medium and ISP medium (developed by Manning, 1975), but they did not report in their results which medium was responsible for successful enrichment. Kamimura *et al* (2001) used a standard minimal medium containing 2.7% NaCl and ferrous sulphate and positive growth was detected visually by the medium turning rusty coloured (due to the formation of ferric hydroxide). Kamimura also used gellan gum plates containing ferrous sulphate and 2.7% NaCl for plating after enrichment in order to purify the isolates and this was the only study reported in the literature that documented the growth of halotolerant bacteria on solid medium. During their study the investigators collected nearly 500 samples and from the enrichments only one isolate was successfully grown and maintained in the laboratory (K. Kamimura, Okayama University, Japan, personal communication 2002). The authors reported observation of iron-oxidation in many of the enrichment cultures but were unable to isolate bacteria from all of these cultures. These reports serve to further highlight the difficulty of isolation of this type of bacteria.

Tilton *et al* (1967a) used a seawater plus thiosulphate medium for enrichment of halotolerant, iron-oxidising bacteria. However, the strains isolated in this way had salinity optima that were well below those of their apparent indigenous environments. In addition, some literature (Cameron et al, 1984) has reported the enrichment and isolation of iron-oxidising bacteria from seawater and marine sediments using media that has been used for the isolation of terrestrial strains, without the addition of NaCl.

It was important to use enrichment media that closely modelled the natural environment from which the samples originated. In doing so, it was hoped that the chances of successfully enriching for and subsequently isolating the target bacteria would be increased. Most of the collected and screened samples came from estuaries which were contaminated by mine tailings including sulphide minerals and a high proportion of pyrite based tailings (D Pirrie, Camborne School of Mines, personal communication, 2000). Therefore there was a high concentration of iron and sulphur containing

compounds in the original samples, which might provide the substrate for the target bacteria. For this reason, pyrite, ferrous iron and tetrathionate were used as nutrient substrates in the subsequent synthetic enrichment media.

The salinity of the sample sites ranged from 24 parts per thousand (2.4%) in estuarine areas with a tidal coverage of seawater to hypersaline conditions of 42 parts per thousand (4.2%) in tidal caves and rock pools. This high salinity may be due to the concentration of salts via evaporation of water from rock pools that may be exposed to the drying effects of the air during low tide. It was therefore decided to use various media of different salinities for enrichment of the target bacteria.

The pH of the samples collected ranged from pH 2.0 (intertidal sediments) to pH 7.0 (seawater samples). However, the majority of the samples were around pH 6.0 – 7.0 probably due to the fact that seawater has a very high buffering capacity. Many of the sediment samples had veins of iron oxide throughout and it was found that these veins were of a lower pH than the surrounding environment. Since the presence of these localised iron oxide veins could reflect oxidation processes by iron-oxidising bacteria, samples of this stained material were used as inocula for some enrichment cultures. The pH values of the original enrichment media were pH 2.0 and pH 5.0, but due to the high number of enrichment cultures it was decided to focus on isolating bacteria at a single pH, so all the subsequent isolation attempts were made at pH 2.0.

All these factors were taken into consideration when developing the programme of enrichment and isolation. A variety of isolation media was used in order to enrich for the target bacteria and growth was checked microscopically every week. The results of the isolation attempts are detailed in this section.

3.2.2 Liquid enrichment and isolation media

Samples of sediment and water from different coastal and estuarine areas were inoculated into different types of enrichment media. The media were designed to mimic the natural environment of the sample source as closely as possible, to increase the chances of enrichment and subsequent isolation of the target bacteria. Sediment samples were transferred into saline and subjected to treatment in a sonic water bath to detach as many of the bacteria as possible from the solid particles and a portion of the sample was used as inoculum for the enrichment medium. The enrichments were incubated at 28°C and 37°C without shaking. For details of enrichment media and resulting isolates see Table 3.1

Every liquid enrichment culture was checked microscopically (using a light microscope at a magnification of X1000) on a weekly basis to determine if cell growth had occurred and those exhibiting detectable growth were sub-cultured successively several times. The pH of these cultures was also monitored and any decrease in pH was noted as this was indicative of the growth of the target acidophiles which produce H_2SO_4 as a product of their metabolism. Enrichment by successive sub-culturing on autotrophic media was used to ensure that the enriched cultures contained the target acidophilic, autotrophic bacteria that were utilising the inorganic substrates provided in the medium. Any colour change in the ferrous iron cultures was noted by comparing them to the control ferrous iron medium and any cultures that exhibited a change in colour were sub-cultured into the same type of medium.

The enrichment cultures were successively diluted at least 5 times before they were colony purified, using the medium outlined in Section 3.8, to isolate pure cultures, for use in subsequent growth, phylogeny and bioleaching experiments.

The pyrite enrichments produced three isolates, two of which originated from the Restronguet Creek and one from the Cligga Head caves, all in medium supplemented with 30 gl^{-1} sea salts at pH 2.0. Ferrous iron enrichments produced one isolate from the mouth of an abandoned tin mine on St. Michael's Mount, in medium supplemented with 20 gl^{-1} sea salts at pH 2.0.

3.2.3 Solid enrichment and isolation media

The enrichment cultures were then plated onto different solid media, including overlay plates, TSM plates and floating membrane culture (see Materials and Methods Sections 2.2.13 and 2.8.5). However, the solid-overlay medium and the TSM plates that were used in this study were not successful in isolating the target bacteria (see Table 3.1 for successful isolation media). Different salt sources and salt concentrations were also tested, again without success. Concentrations of gelling agents were also varied to ascertain whether this parameter was important for the growth of the target bacteria but again no growth was observed on these solid media. For all these experiments the solid isolation plates were incubated at both 28°C and 37°C.

However, some success was achieved by using a floating nylon membrane method, which provided a solid substratum for growth of bacteria without the need for potentially toxic gelling agents. Also, medium and waste products could move freely through the membrane, therefore providing available nutrients for growth of bacteria. Ferrous iron medium was used in these plates with 30 gl^{-1} sea salts and no organic substrate was added to this medium. Figures. 3.1 a, b and c show the growth of some isolates on these membrane culture plates. However, these plates and membranes dried out very quickly and had to be topped up every day with fresh medium to maintain a humid environment for growth. Rust coloured, raised colonies appeared after 3 – 4 weeks of incubation (Figures 3.1 a, b and c) and colony formation was quicker when incubated at 37 °C than at 28 °C. Samples of these colonies were examined microscopically at X1000 magnification and rod-shaped cells were observed (data not shown). The rod-shaped bacteria were Gram-negative and ranged in size from 1 μm to 5 μm in length and 0.1 μm to 0.3 μm in width. Colonies were picked from these membrane cultures and were inoculated into fresh ferrous iron liquid medium of the same composition as the membrane medium, but microscopic examination revealed that after even prolonged incubation no growth occurred. However, colonies could be successfully sub-cultured onto membrane culture plates without loss of viability.

The liquid enrichment cultures that exhibited sustained growth during serial sub-culturing were inoculated onto the surface of the nylon membrane culture plates and rust coloured colonies were observed after about 4 weeks. Samples of the colonies were

examined under the microscope and the three isolates from the pyrite liquid medium were found to be Gram-positive, non-motile, rods, and the isolate from the St. Michael's enrichment culture comprised Gram-negative motile rods. The St. Michael's culture did not grow in liquid pyrite medium.

The isolates were designated 4G, 5C and Cligga, St. Mics b and Calenick b (according to an identification system that referred to the original sample identification and enrichment medium)

The Calenick (Calenick b) and Tresillian enrichment cultures did not successfully grow in ferrous iron, pyrite or tetrathionate liquid medium and therefore it was impossible to efficiently maintain these cultures. The Gram-negative isolate from St. Michael's (St. Mics b) only grew in ferrous iron saline medium very slowly and not at all on pyrite or tetrathionate. Genomic DNA was extracted from this culture and the phylogeny assessed by sequencing of the 16S rDNA region. It was found that the partial 16S rRNA gene from St. Mics showed highest identity with *Marinobacter* spp. of 92% suggesting that this strain represented a novel species within the genus *Marinobacter*, however, no further work was carried-out with this strain. Due to the prolonged period of incubation required to grow the Calenick b and Tresillian isolates, it was decided to further characterise the isolates 4G, 5C and Cligga as they exhibited good growth in pyrite medium with 30 gl^{-1} sea salt.

Figure 3.1a

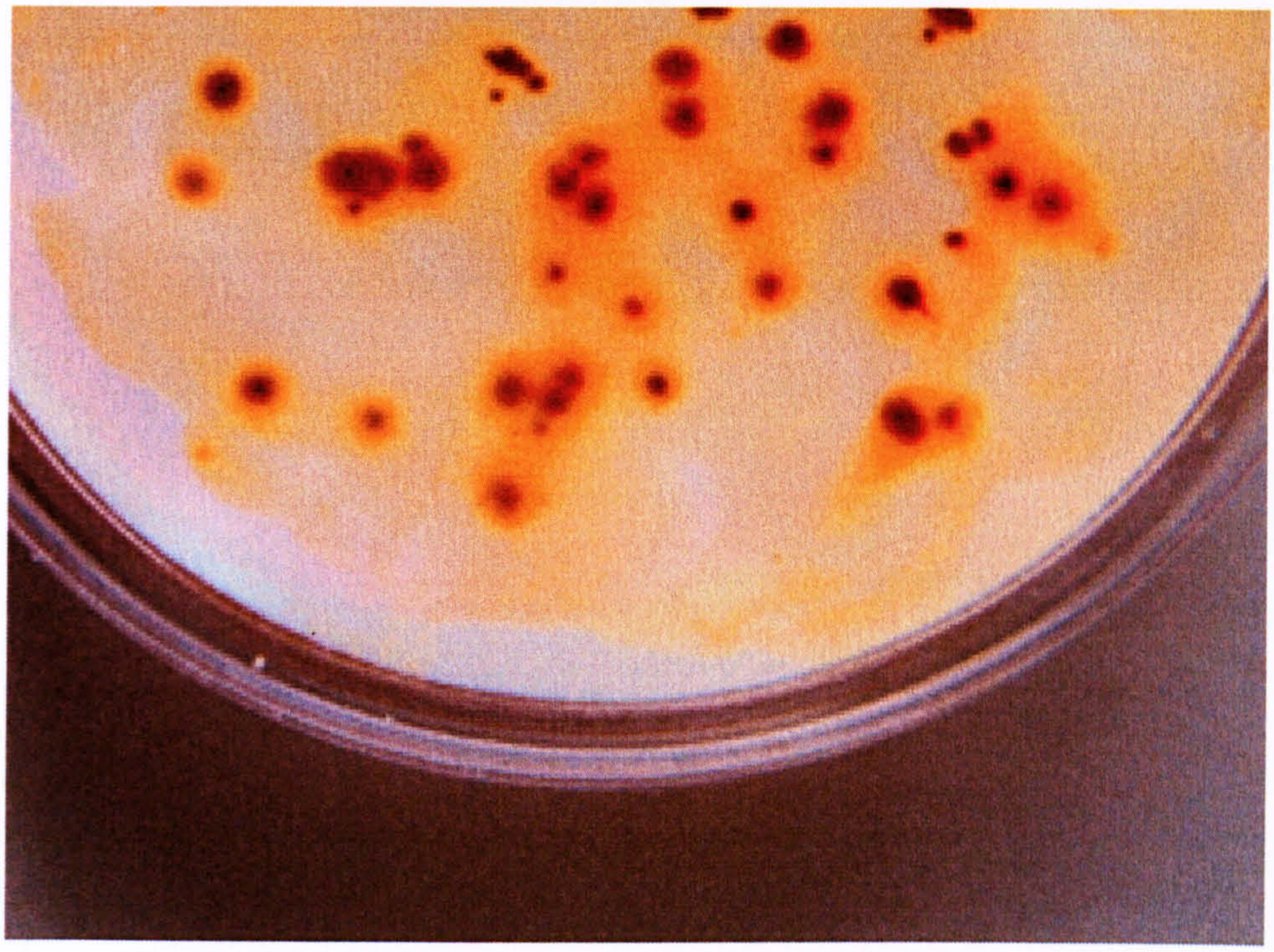


Figure 3.1b

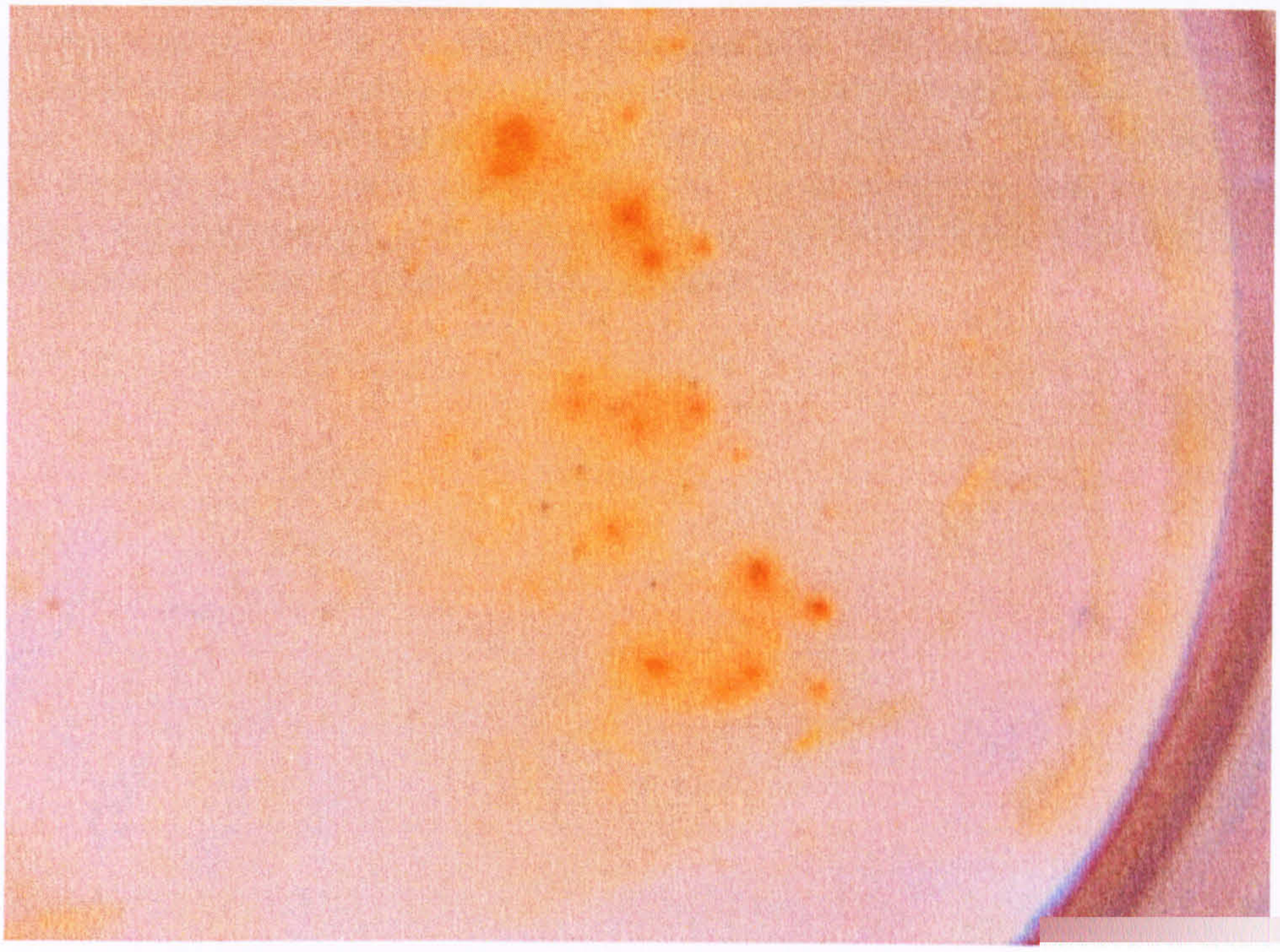
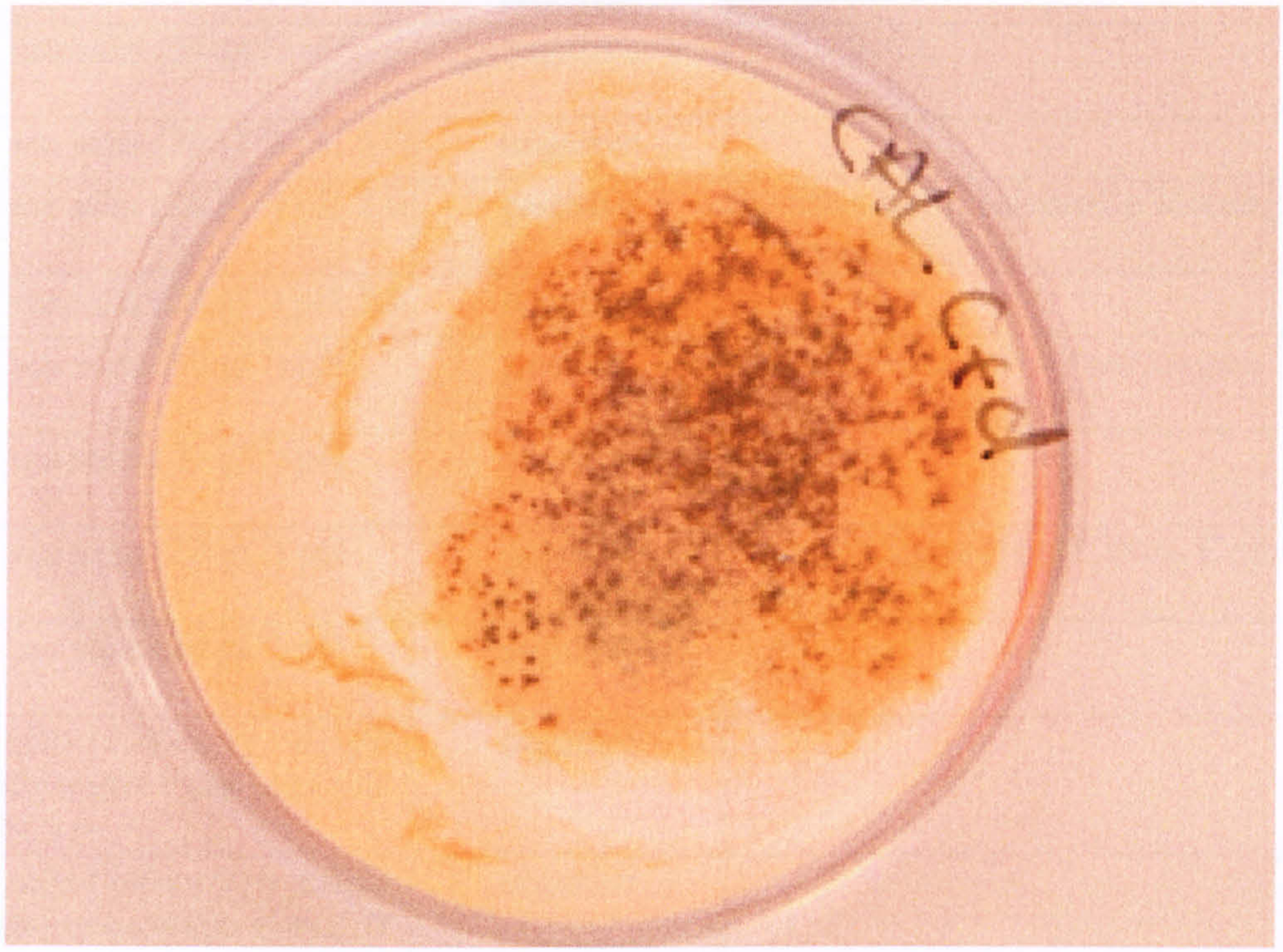


Figure 3.1c



Figures 3.1 a, b and c: Colony formation on nylon membrane culture vessels. Figure 3.1 a shows colonies from an *At. ferrooxidans* (ATCC 23270) culture, 3.1b shows an enrichment culture from the Tresillian River and 3.1c shows an enrichment culture from a Calenick Creek sample. The colonies formed were rust coloured and were surrounded with a lighter orange coloured halo. Colonies were picked, examined microscopically and then gram stained. The colonies that are shown in Figure 3.1a were motile rods and Gram-negative, this was expected as this plate was inoculated with *At. ferrooxidans*. Colonies from Figures 3.1 b and c. also comprised Gram negative highly motile rods.

Table 3.1 Media used for the enrichment of halotolerant, iron-oxidising acidophilic bacteria (see Materials and Methods section 2.2.3 for media recipes).

Isolation medium	Enrichment of isolates	Final isolate obtained
FM + sea salts (30 gl^{-1}) pH 2.0	negative	
FM + sea salts (30 gl^{-1}) pH 5.0	negative	
FM + sea salts (20 gl^{-1}) pH 2.0	**positive**	St. Mics b (St. Michael's Mount, Cornwall UK)
FM + sea salts (20 gl^{-1}) pH 5.0	negative	
PM + sea salts (30 gl^{-1}) pH 2.0	**positive**	Isolates 4G and 5C (Restronguet Creek, Cornwall, UK)
PM + sea salts (30 gl^{-1}) pH 5.0	negative	
PM + sea salts (20 gl^{-1}) pH 2.0	**positive**	Cligga (Cligga Head Caves, Cornwall, UK)
PM + sea salts (20 gl^{-1}) pH 5.0	negative	
Basal salts (10% v/v) + dH_2O + tetrathionate + sea salts (30 gl^{-1}) pH 2.0	negative	
Basal salts (10% v/v) + dH_2O + tetrathionate + sea salts (30 gl^{-1}) pH 5.0	negative	
Basal salts (10% v/v) + dH_2O + tetrathionate + sea salts (20 gl^{-1}) pH 2.0	negative	
Basal salts (10% v/v) + dH_2O + tetrathionate + sea salts (20 gl^{-1}) pH 5.0	negative	
Estuarine enrichment medium	negative	
Solid overlay medium + sea salts (30 gl^{-1}), pH 2.O	negative	
Solid overlay medium + sea salts (20 gl^{-1}), pH 2.O	negative	
TSM solid medium + sea salts (30 gl^{-1}), pH 2.O	negative	
TSM solid medium + sea salts (20 gl^{-1}), pH 2.O	negative	
Floating membrane method + sea salts (30 gl^{-1}), pH 2.O	**positive**	Isolates 4G + 5C (Restronguet Creek, Cornwall, UK)
Floating membrane method + sea salts (20 gl^{-1}), pH 2.O	**positive**	Tresillian l (Tresillian River, Cornwall, UK), Calenick Creek b (Calenick Creek, Cornwall, UK)

3.3 Morphology of the isolated bacterial strains

3.3.1 Introduction

Morphological features are important in the initial tentative identification of new bacterial isolates and together with molecular and biochemical data, are important in determining phylogeny of potentially novel bacteria. One of the most widely characterised iron-oxidising, acidophilic bacterial species *At. ferrooxidans* is a Gram-negative rod shaped bacterium of between 1 –3 μm in length and 0.1 – 0.5 μl in width (Rawlings *et al*, 1999). Another, *L. ferrooxidans* is a gram negative, spiral shaped bacterium of between 1 - 2 μm long and 0.1 – 0.3 μm wide (Rawlings *et al*, 1999). Other acidophiles include *Sulfobacillus spp.* which are gram positive rods that form endospores which can usually be viewed under the light microscope at a magnification of X1000 (Karavajko *et al*, 1990 and Tourova *et al*, 1994)

3.3.2 Morphology

The morphology and size of the environmentally isolated bacteria were assessed using a light microscope with the oil immersion lens at a magnification of X1000. The data in Table 3.2 summarises the morphological characteristics of the isolated bacteria. All of the isolated bacteria were long rods that sometimes were joined terminally, occurring in chains of up to 5 bacteria (See Figures 3.2a, b, c and d for pictures of cells). The cells were shorter, wider and sometimes irregularly shaped when grown on solid agarose medium and when exposed to stress factors such as excessive heat (above 45°C). Figure 3.2d shows a Scanning Electron Microscope Image of bacterium 4G grown on heterotrophic salt medium, this image shows clearly the rod-shaped morphology of the bacterium.

Figure 3.2a

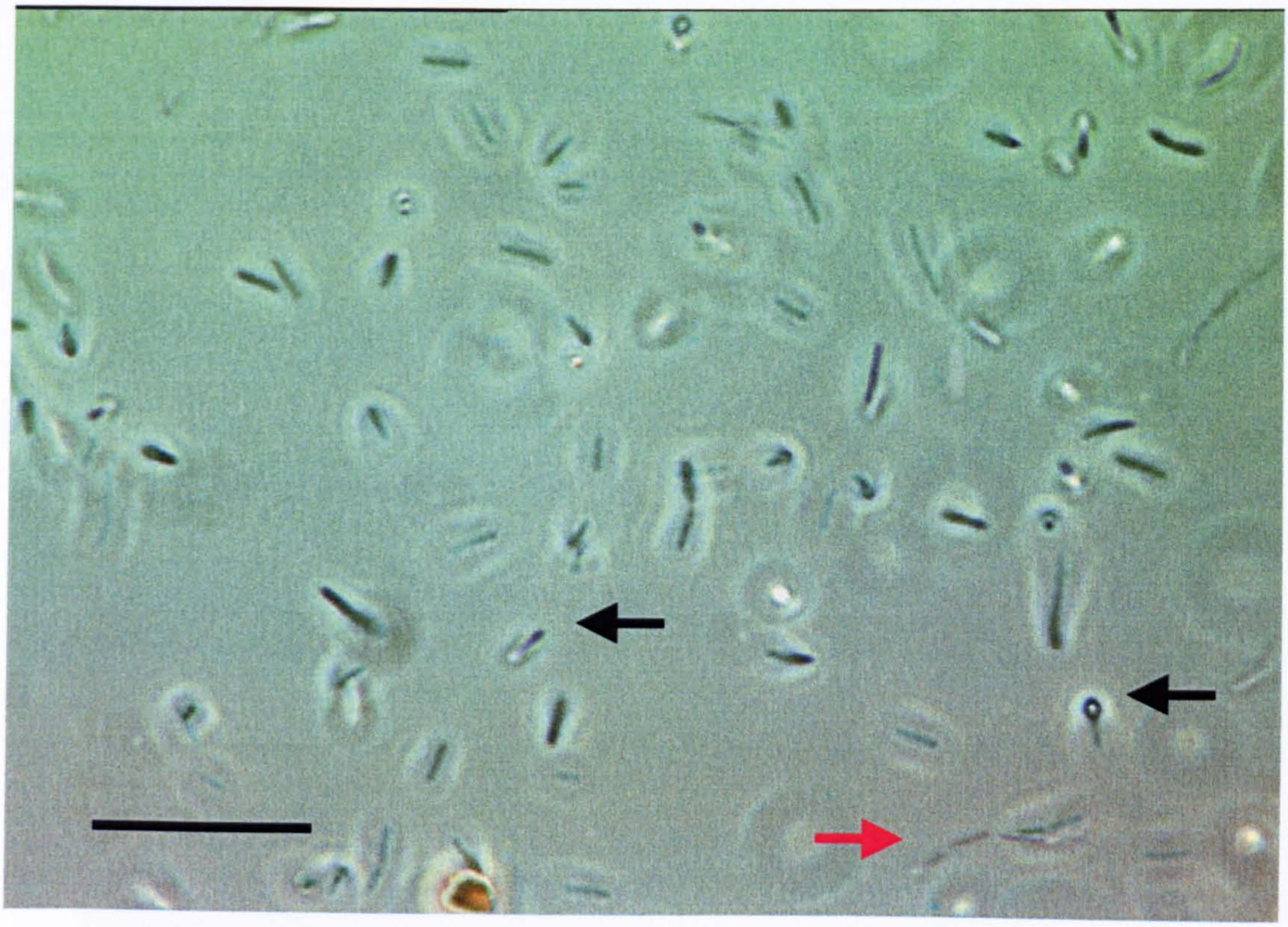


Figure 3.2b

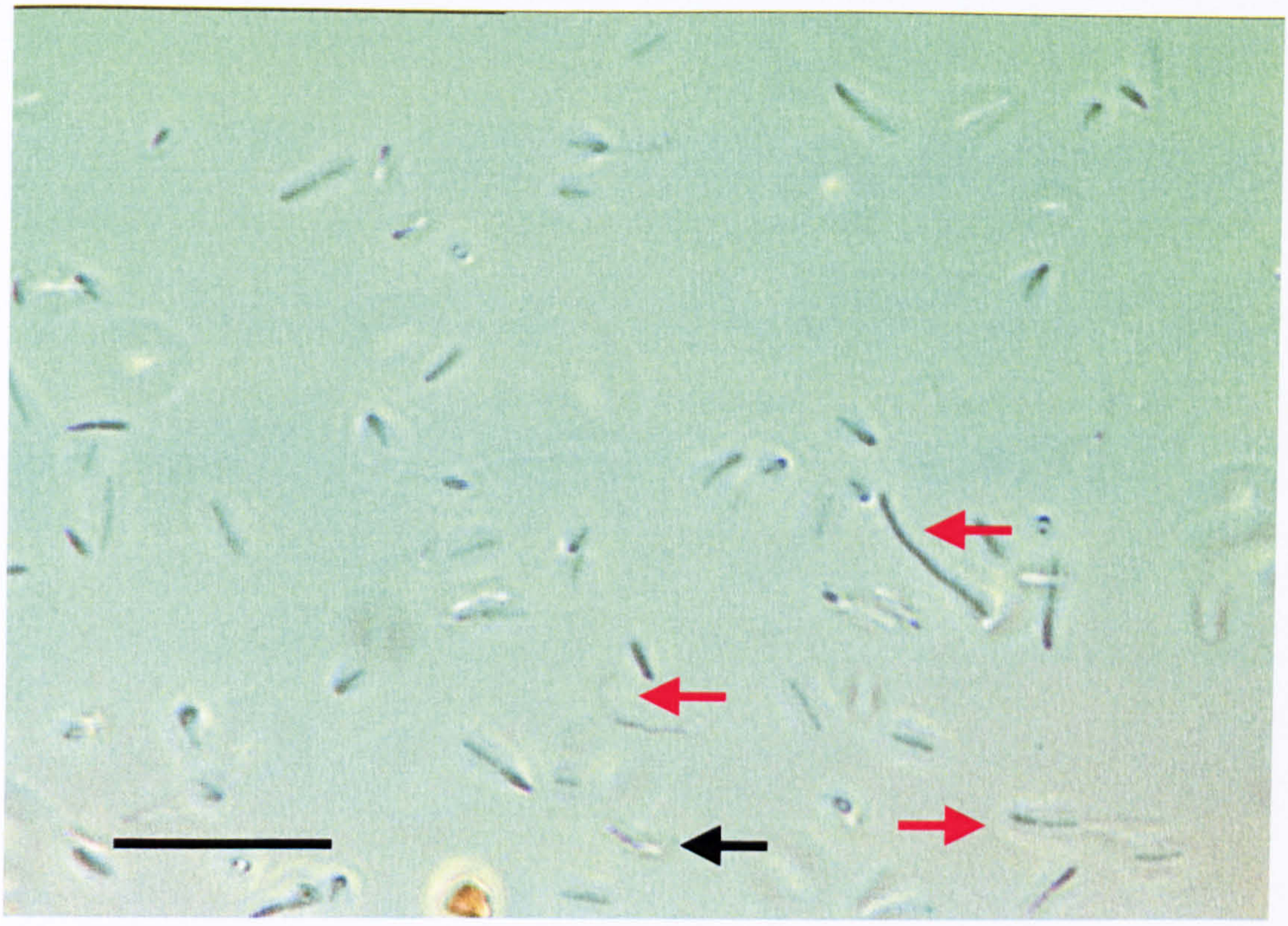


Figure 3.2c

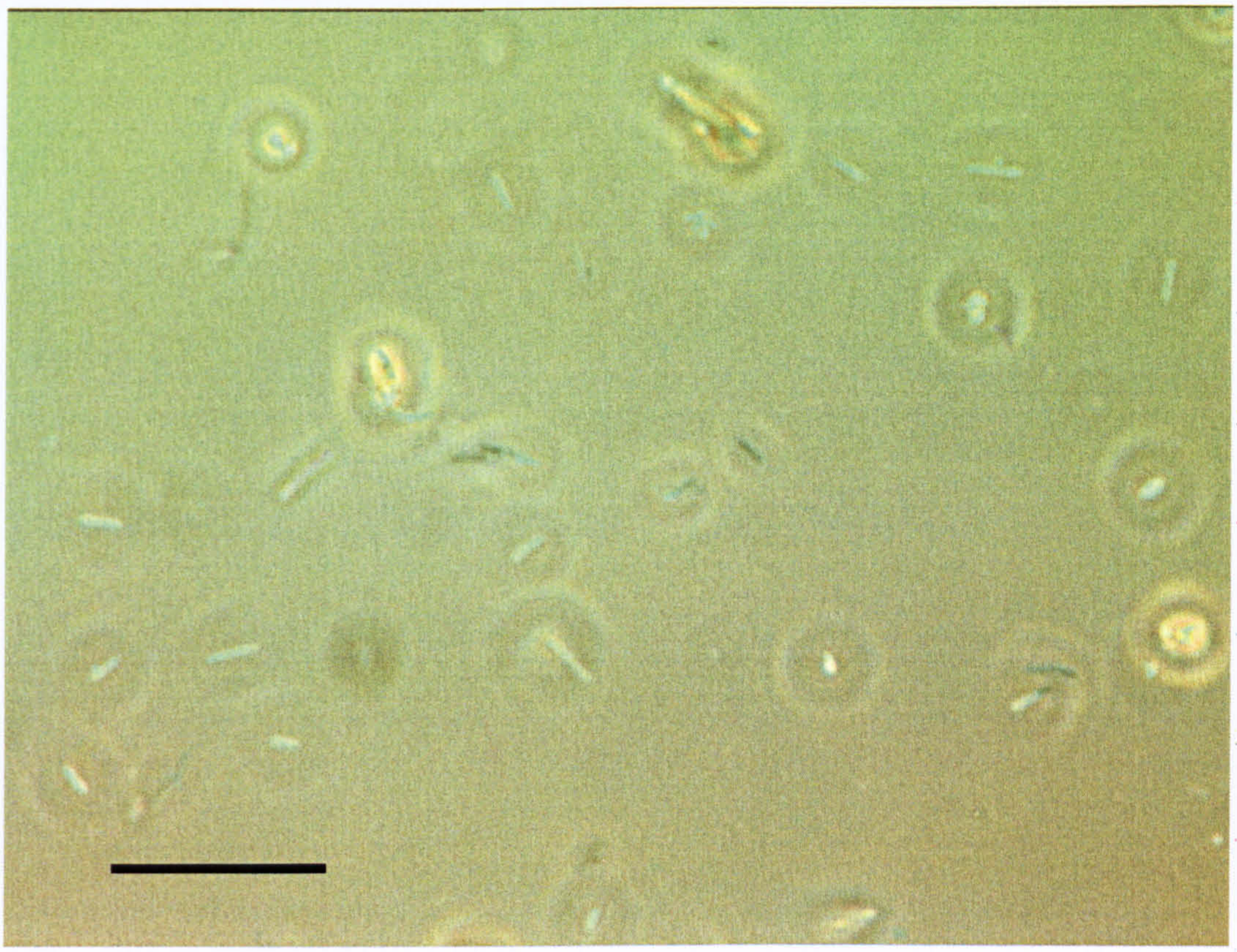
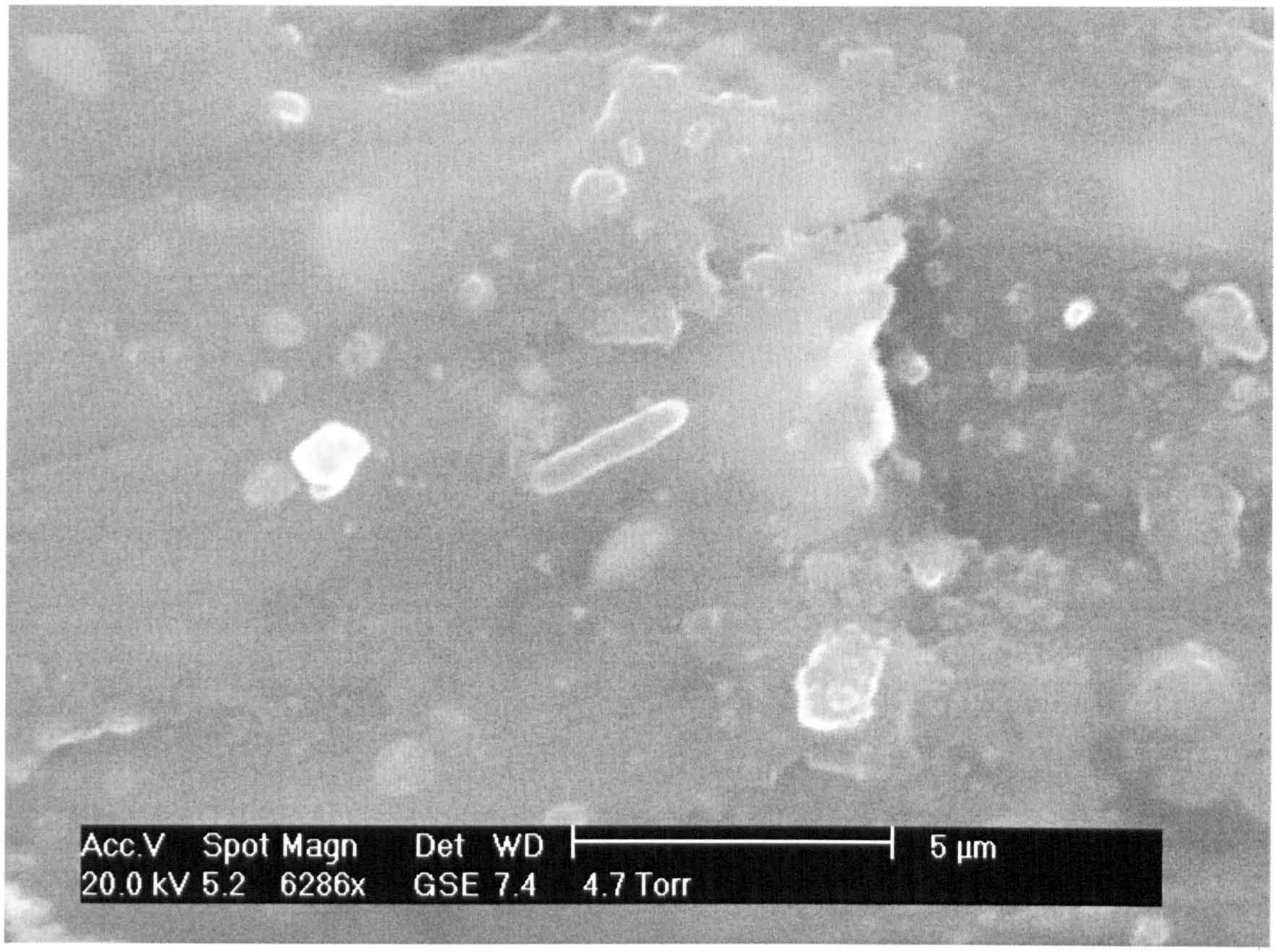


Figure 3.2d



Figures 3.2 a, b and c show cultures of the isolated bacteria grown for 10 days on Las Cruces Spanish copper mineral ore, at a magnification of X1000. Figure 3.2a shows strain 4G, Figure 3.2b shows strain 5C and Figure 3.2c shows strain Cligga. The isolates are all gram positive, non-motile long rods. The formation of terminal spores can be seen on Figures 3.2a and 3.2b, characterised by an oval shaped swelling at one end of the cell (identified by the black arrows). Chains of cells of up to 5 bacteria occur in these cultures regularly (identified by the red arrows). The scale bar shows 5 µm. Figure 3.2d shows a Scanning Electron Micrograph of a strain 4G bacterium at a magnification of X6286. This bacterium is approximately 2.5µm long and 0.5 µm wide.

Table 4.2 Morphology of isolated bacteria

Isolate	Gram stain	Shape	Length (µm)	Width (µm)	Position of spores
4G	+ve	long rods	1 - 5	0.2 – 0.5	terminal
5C	+ve	long rods	1 - 5	0.2 – 0.4	terminal
Cligga	+ve	long rods	1 - 4	0.2 – 0.4	terminal

3.4 Growth of the isolated bacteria at different concentrations of sea salts

3.4.1 Introduction

Seawater is a complex mixture of many different compounds. Salinity varies from area to area but the relative proportions of ions remain relatively constant. Although NaCl is the major constituent of seawater, KCl and sulphate salts are other major constituents (Brown *et al*, 2002). All the dissolved ions in seawater together make up the final salinity of seawater. The average salinity of seawater is 35 parts per thousand and this is comprised of many different elements. Major constituents of seawater are those that occur in concentrations greater than one part per million (ppm) by weight, and account for 99.9% of the dissolved salts in the oceans. The minor and trace constituents are all of the other elements in seawater.

Most studies on the extent of halotolerance of autotrophic bacteria are carried out with NaCl as the main source of salt; very few have used seawater, either artificial or natural, in their studies. Some studies on heterotrophic marine bacteria have suggested that potassium chloride may play some part in the ability of bacteria to withstand the osmotic stress exerted by elevated NaCl concentrations. Reichelt & Baumann (1974) found that some bacteria accumulate KCl intracellularly in response to elevated concentrations of NaCl, and this offers a protective effect from osmotic stress.

The salinities of the environments from which the test strains were originally isolated varied from 2.4 % to 4.2 %. The sample from which 4G was isolated had 3.2%, 5C 3.5% and Cligga 4.2% salinity. Some studies report that salinity optima of marine or halotolerant isolates are much lower than those of their natural environments (Smith & Finazzo, 1981). *T. prosperus* displayed optimum growth in the absence of NaCl, despite tolerating up to 3.5 % NaCl (Huber & Stetter, 1989). *T. intermedius* was isolated from salt marsh sediments with an interstitial salinity of 3 % but optimum growth was recorded at a salinity of 1 % (Smith & Finazzo, 1981). The authors concluded that *T. intermedius* grew at its maximal growth rate only rarely in nature. Kamimura *et al.* (2001) reported that strain KU2-11, an iron- and sulphur-oxidising bacterium isolated from open seawater that obligately required NaCl for growth, had an optimum salt concentration for growth of 2 %. *T. halophilus*, is a halophilic, sulphur-oxidising bacterium isolated from a hypersaline lake, and had a growth salinity

optimum of 5.8 % but it was noted that this microorganism is not an acidophile, having a pH optimum for growth of pH 7.0 (Wood and Kelly, 1991).

The growth responses of the three isolated bacterial strains at different salinities were evaluated (at concentrations of sea salts up to 30 gl^{-1}) to determine the optimum salinity for bioleaching experiments and other growth experiments.

3.4.2 Growth of strain 4G at different levels of salinity

Figure 3.3 shows the growth of isolate 4G in media containing different concentrations of sea salts. It can be seen that the rate of growth of 4G was higher in the medium with 30 gl^{-1} sea salts than in the medium with 20 gl^{-1}, 10 gl^{-1} or with no salt source added. This result confirms that the optimum growth salinity of 4G (of those salinities tested) was 30 gl^{-1} sea salts, which is close to that of the environment from which this strain was isolated (interstitial salinity of water in the sediment sample was 32 gl^{-1}). Growth rate constant increases with increasing salt concentration and was at its lowest with no added salt in the medium (Figure 3.6). The highest cell number attained during growth with 30 gl^{-1} sea salt was $5.37x10^7$ cells ml^{-1} after 6 days of growth. The isolate exhibited lag periods of around two days at 30 gl^{-1} and 20 gl^{-1} sea salts and lag periods of around 3 days at 10 gl^{-1} sea salts and with no added salts.

3.4.3 Growth of strain 5C at different levels of salinity

Growth of isolate 5C in medium containing different concentrations of sea salts is shown in Figure 3.4. The optimum growth salinity (within the tested salinity range) of 5C is 30 gl^{-1} sea salts as the rate of growth of 5C was higher in the medium with 30 gl^{-1} sea salts than in the medium with 20 gl^{-1}, 10 gl^{-1} or with no salt source added (Figure 3.6). This bacterium was isolated from a sediment sample that had an interstitial salinity of 35 gl^{-1} and this indicates that this bacterium is probably well adapted to growth at this salinity. Cultures of this bacterium exhibited lag periods of around one day at 30 gl^{-1} and 20 gl^{-1} sea salts and lag periods of around 4-5 days at 10 gl^{-1} sea salts and with no added salts. Cell number reached its highest level during growth in 30 gl^{-1} sea salt conditions was $1.84x10^7$ cells ml^{-1} after 5 days of growth.

3.4.4 Growth of strain Cligga at different levels of salinity

It can be seen from Figure 3.5 that the rate of growth of Cligga was highest in medium with 30 gl^{-1} sea salts as compared to the other levels tested, indicating that 30 gl-1 sea salts is the optimum growth salinity for this strain (within the salinity range tested). This salinity is less than that of the environment from which this strain was isolated (42 gl^{-1}), however, this strain may exhibit higher growth rates at higher salinities. Strain Cligga exhibited short lag periods of less than one day at 30 gl^{-1} sea salts and one day at 20 gl^{-1} and 10 gl^{-1} sea salts and a lag period of around 2 days with no added sea salts. The growth rate constant of this strain was at its lowest with no added sea salts (Figure 3.6) and the highest cell number attained during growth in 30 gl^{-1} sea salt conditions was $1.49x10^7$ cells ml^{-1} after 4 days of growth.

3.4.5 Growth of isolates 4G, 5C and Cligga in medium containing NaCl as compared to sea salts

Figure 3.7 shows the growth of the three salt-tolerant bacteria in pyrite medium containing 30 gl^{-1} NaCl. It was found that growth in pyrite medium with 30 gl^{-1} NaCl was much slower than growth in pyrite medium with 30 gl^{-1} sea salts. The exponential growth rate constant of 4G was 0.42, 5C was 0.24, and Cligga was 0.21 day^{-1}. Isolate 4G had a growth rate constant in sea salts medium that was 2.5 times that in NaCl medium and isolate 5C had a growth rate in sea salts medium that was 4.1 times that in NaCl medium. Also, isolate Cligga had a growth rate in sea salts medium that was 3.8 times that in NaCl medium.

It should be noted that growth on medium without any salt added shows that none of the isolates has an absolute requirement for a salt source for growth.

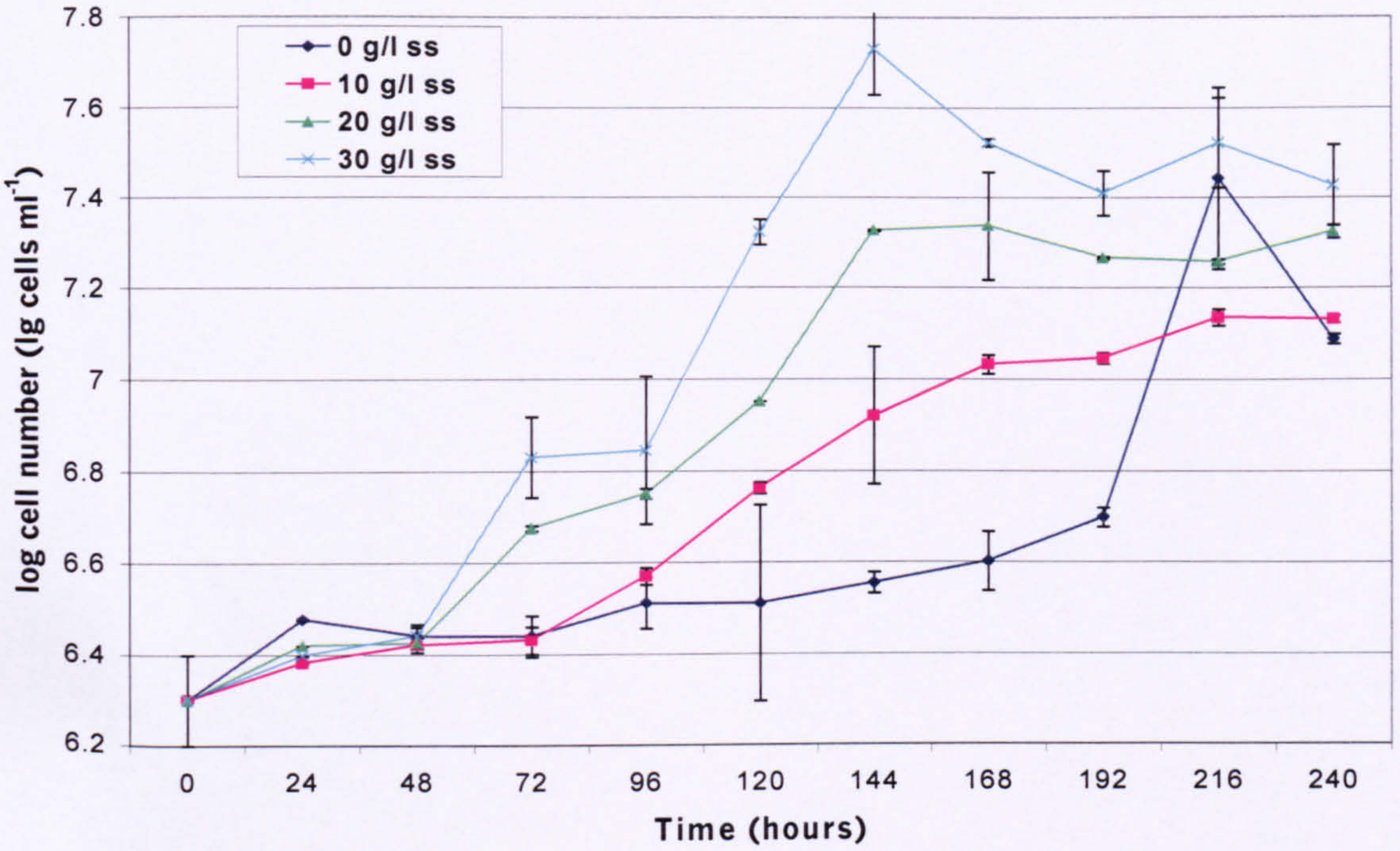


Figure 3.3 Growth of isolate 4G in PM with 0, 10, 20 and 30 gl^{-1} added sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

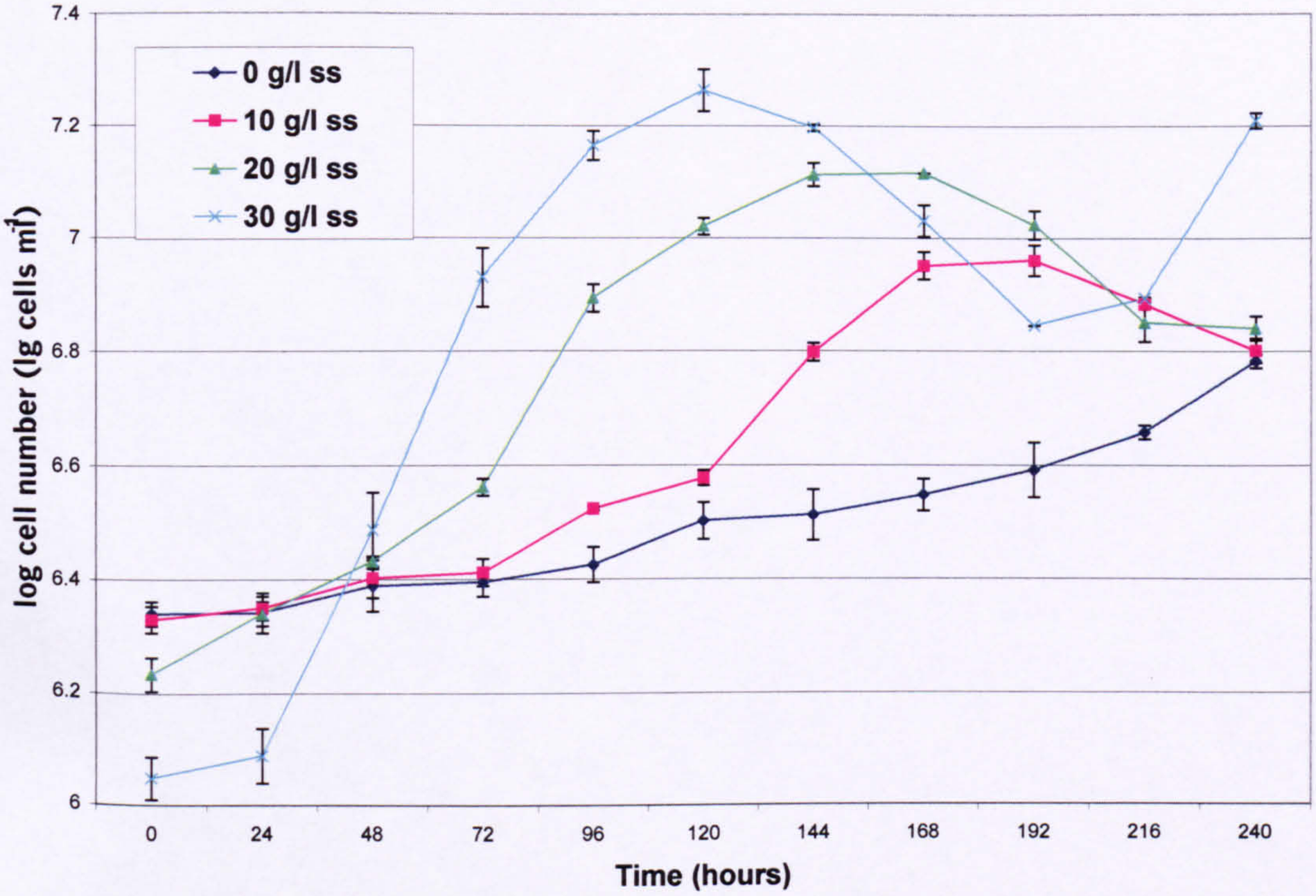


Figure 3.4 Growth of isolate 5C in PM with 0, 10, 20 and 30 gl^{-1} added sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

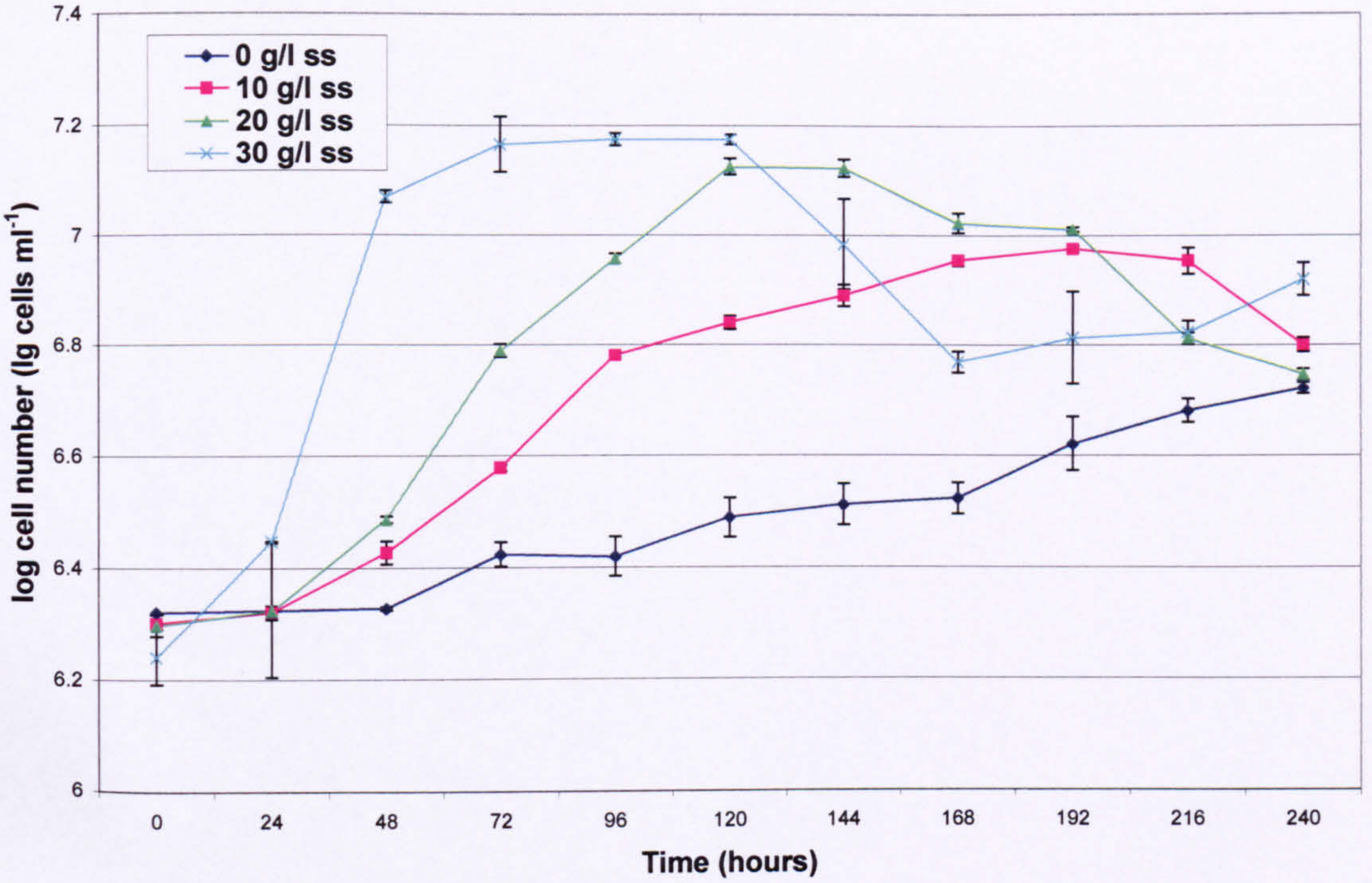


Figure 3.5 Growth of isolate Cligga in PM with 0, 10, 20 and 30 gl^{-1} added sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

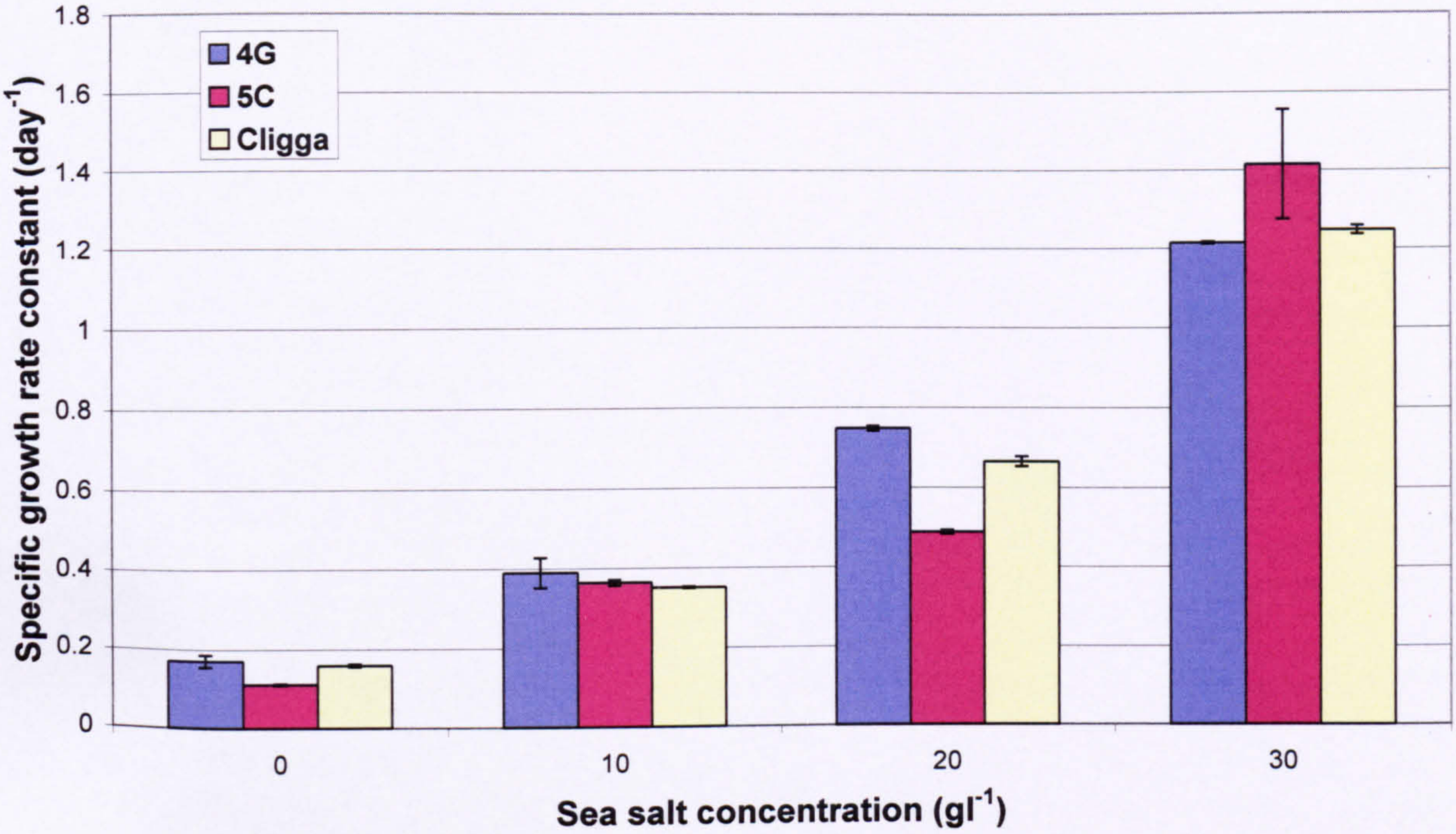


Figure 3.6 Growth rate constants of isolates 4G, 5C and Cligga in PSM of different salinity. Each datum bar represents the mean ± standard deviation of duplicate cultures.

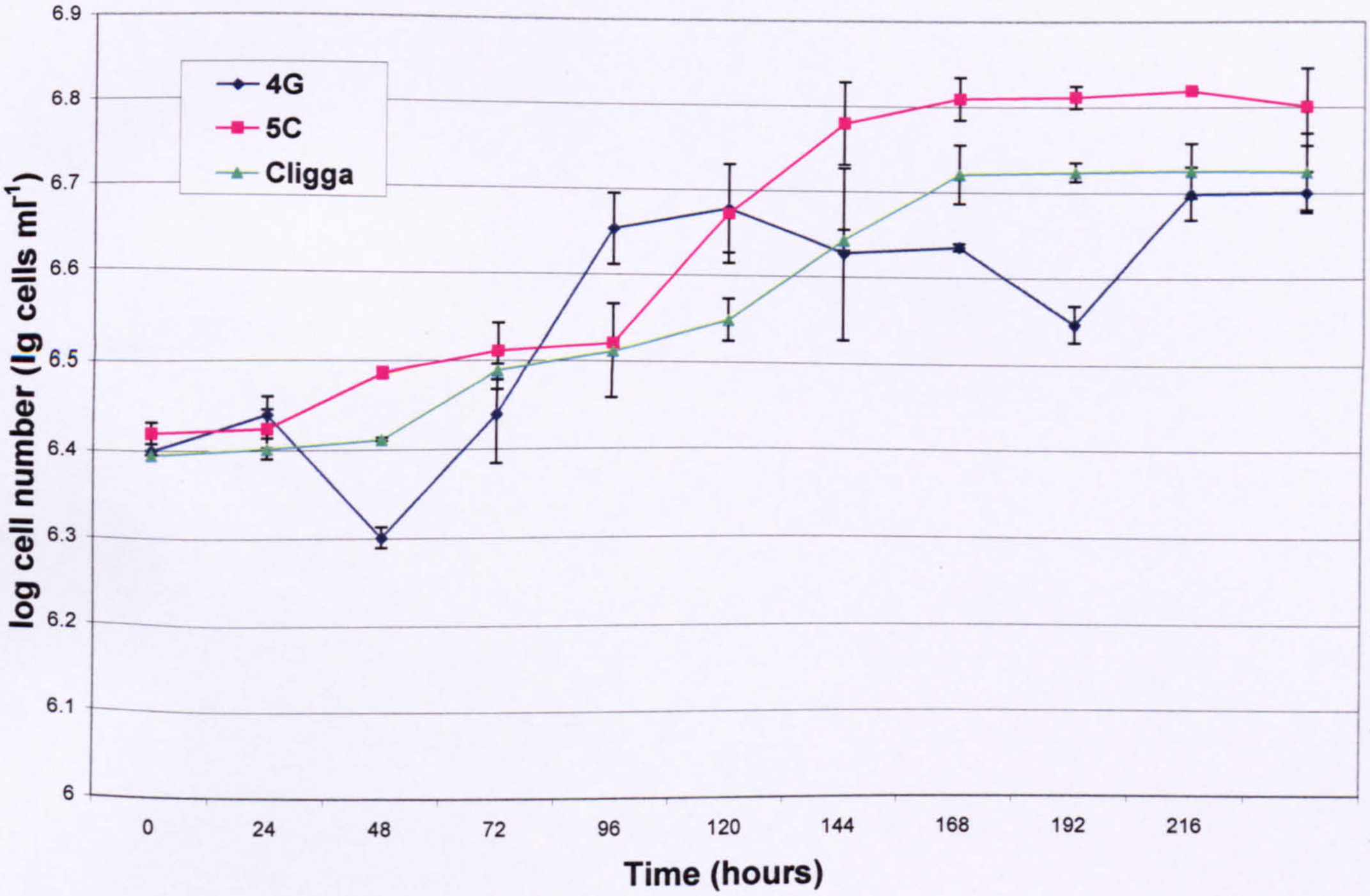


Figure 3.7 Growth of environmental isolates 4G, 5C and Cligga in PM with 30 gl^{-1} NaCl. Each datum point represents the mean ± standard deviation of duplicate cultures.

3.5 Growth of the isolated bacteria under mixotophic and heterotrophic conditions

3.5.1 Introduction

Many gram-positive, acidophilic, iron oxidising bacteria grow using different modes of nutrition. For example *Sulfobacillus sp.* can grow autotrophically, mixotrophically and heterotrophically (Norris *et al*, 1996). There have also been reports of increasing growth rate constants and higher overall cell yield by augmentation of autotrophic growth medium with organic substrates such as yeast extract. (Yahya & Johnson, 2002). These increases in growth rate constants may be due to the lower amount of energy expended by the bacteria in using organic carbon sources than fixing atmospheric carbon, thereby leaving more energy for growth and metabolic processes. The growth of many iron-oxidising bacteria is generally inhibited by high amounts of organic material in the medium and therefore, it was decided to assess and compare growth responses of the isolates in mixotrophic and heterotrophic media. PSM medium was used for mixotrophic growth and YSM medium used for heterotrophic growth (see section 2.2.3 for media compositions).

3.5.2 Growth of strain 4G under mixotrophic and heterotrophic conditions

Figure 3.8 shows that isolate 4G exhibited a long lag phase when grown on heterotrophic medium, and that it took 4G four days to reach the beginning of exponential phase on this medium. However, the rate of growth during log phase was high and stationary phase was reached within a day. Exponential growth on mixotrophic medium occurred over a longer time (at a slower rate of growth), but the lag phase on this medium was only two days. The mean generation time during exponential growth was 19.77 hours on the mixotrophic medium and 7.04 hours on the heterotrophic medium, illustrating the dramatic difference in growth rate on the two different types of medium.

3.5.3 Growth of strain 5C under mixotrophic and heterotrophic conditions

It can be seen from Figure 3.9 that, in contrast to isolate 4G, isolate 5C had a longer lag phase when grown in mixotrophic conditions than in heterotrophic conditions. 5C exhibited a lag phase of two days under mixotrophic growth conditions whereas cultures grown on heterotrophic medium reached the exponential phase within one day of inoculation. The exponential growth rate of 5C in heterotrophic medium was more than twice that in mixotrophic medium, and stationary phase was maintained for a longer time in hetertrophic medium than in mixotrophic medium. Even though the inoculum of 5C cells into heterotrophic medium was less than that into mixotrophic medium, maximum cell numbers were higher in the heterotrophic medium.

3.5.4 Growth of strain Cligga under mixotrophic and heterotrophic conditions

Isolate Cligga exhibited a long lag phase in heterotrophic medium (four days) as compared to mixotrophic conditions (one day) as shown in Figure 3.10. However, as was observed with cultures of isolate 4G, the exponential growth rate in heterotrophic medium was much higher than that in mixotrophic medium. Higher final cell numbers were reached in heterotrophic medium despite having a lower initial cell inoculum. The highest cell numbers observed in mixotrophic medium were 1.49×10^7 cells ml^{-1} and occurred after four days of growth, compared to 3.01×10^7 cells ml^{-1} in heterotrophic medium.

3.5.5 Specific growth rate constants of strains 4G, 5C and Cligga

Figure 3.11 shows that growth rate constants of all three environmental isolates were much higher in heterotrophic medium than in mixotrophic medium. During heterotrophic growth, mean generation times of 4G, 5C and Cligga were 7.04, 6.76 and 7.69 hours respectively. These were extremely short generation times as compared to mixotrophic growth which where 19.77, 16.69 and 19.25 hours respectively.

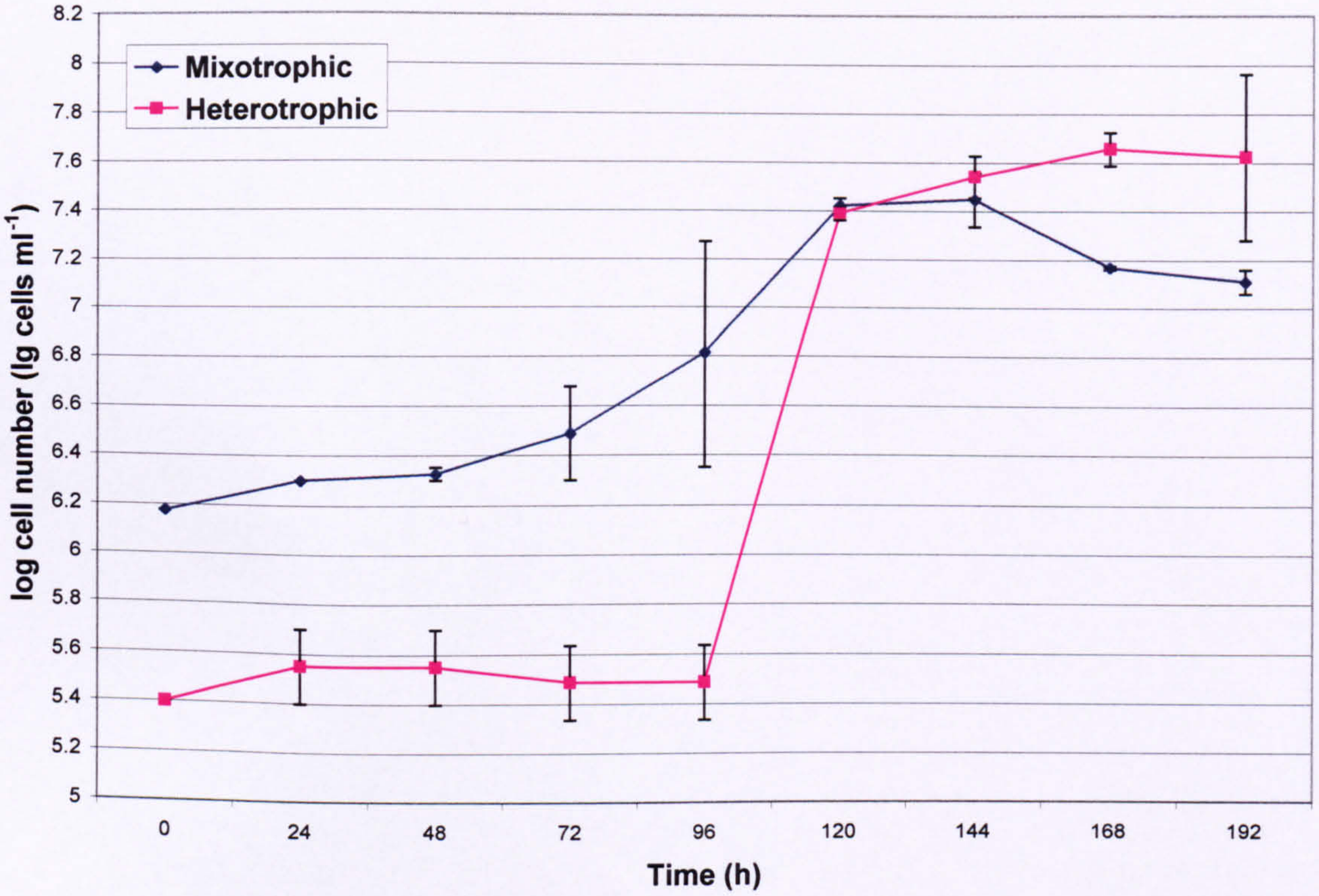


Figure 3.8 Comparison of growth of isolate 4G in PSM (mixotrophic conditions) and YSM (heterotrophic conditions). Each datum point represents the mean ± standard deviation of duplicate cultures.

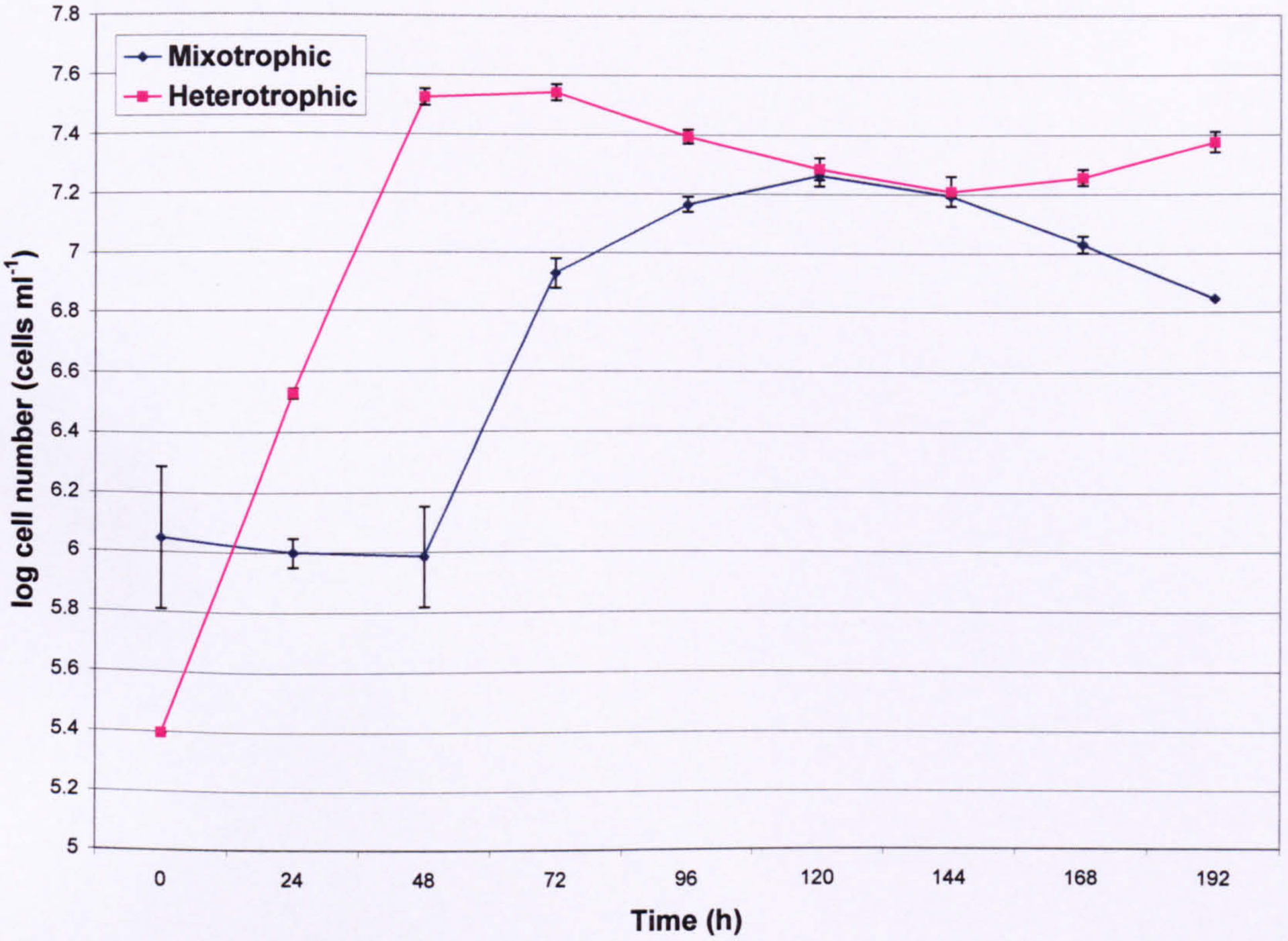


Figure 3.9 Comparison of growth of isolate 5C in PSM (mixotrophic conditions) and YSM (heterotrophic conditions). Each datum point represents the mean ± standard deviation of duplicate cultures.

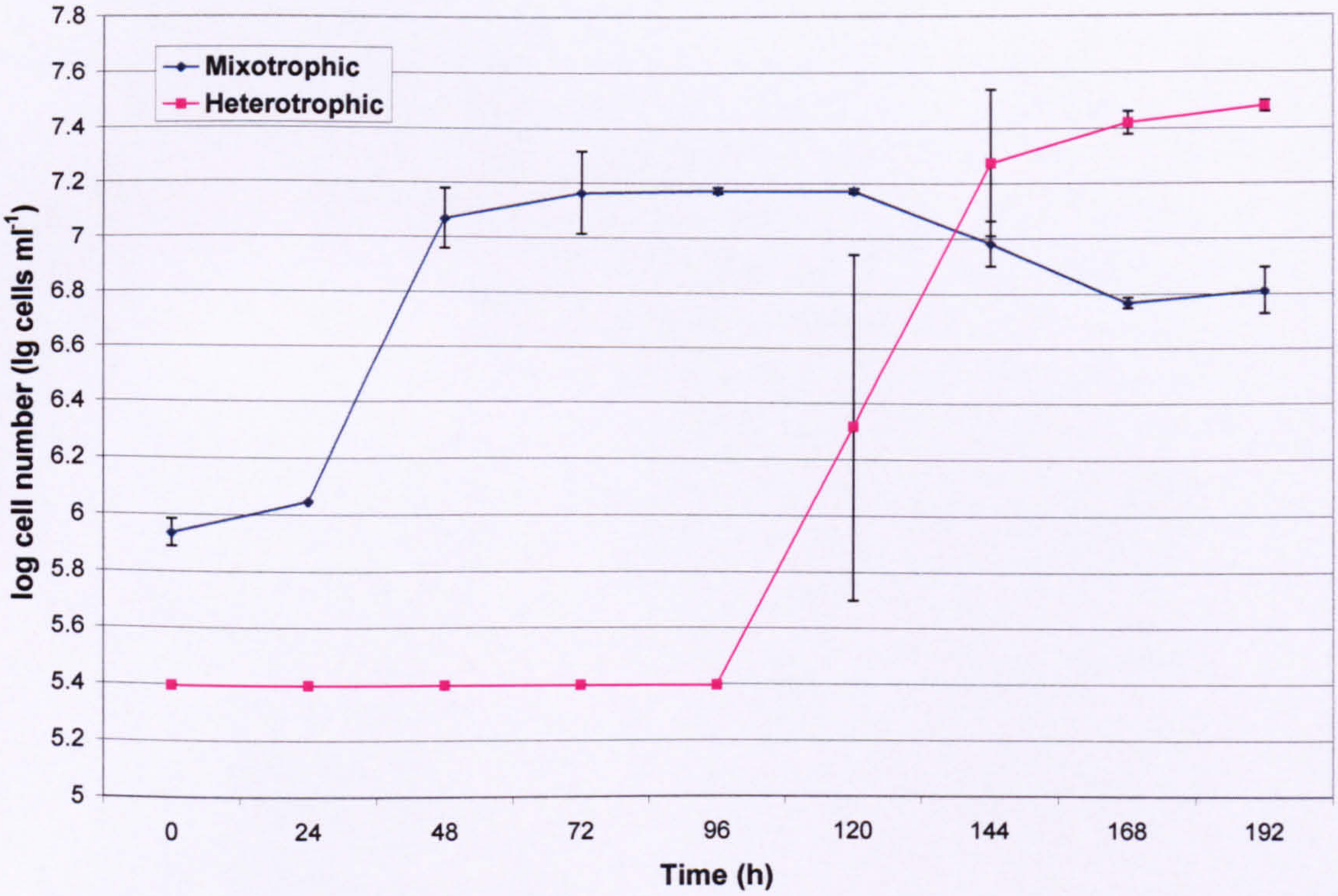


Figure 3.10 Comparison of growth of isolate Cligga in PSM (mixotrophic conditions) and YSM (heterotrophic conditions). Each datum point represents the mean ± standard deviation of duplicate cultures.

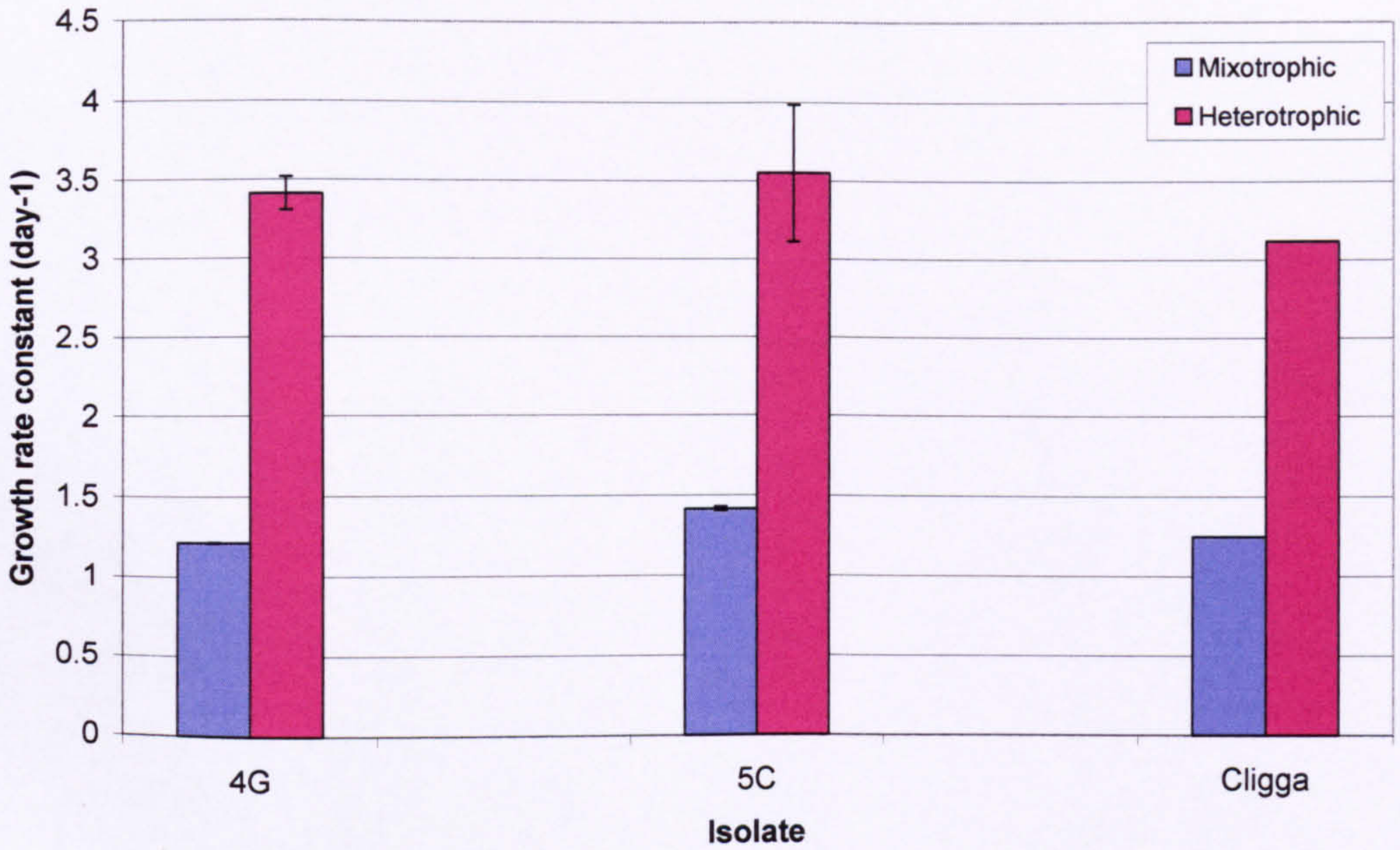


Figure 3.11 Growth rate constants of isolates 4G, 5C and Cligga when grown in PSM (mixotrophic conditions) and YSM (heterotrophic conditions). Each datum bar represents the mean ± standard deviation of duplicate cultures.

3.6 Optimum temperature for growth of the isolated bacteria

3.6.1 Introduction

Iron-oxidising bacteria have been isolated from areas of different temperature environments. These range from low temperature soil environments and marine areas (this study) and naturally heated bioleaching heaps and coal spoilage heaps (Langdahl & Ingvorsen, 1997), to high temperature geysers (Atkinson *et al*, 2000 and Johnson *et al*, 1997), yielding psychrophilic, mesophilic and thermophilic isolated bacteria. However, optimum growth temperatures of isolates usually differ from the normal temperature of the immediate environment from which they were isolated.

Bioleaching at high temperature may potentially accelerate leaching of metals from pyritic ore, however, the costs of heating during such processes can make the process economically unviable. The metabolic processes of bacterial consortia in spoilage heaps and bioleaching heaps, naturally elevate the temperature of such environments, and temperatures can range from 30°C to 50°C (Bustos *et al*, 1999 and Rawlings, 2002). It is therefore desirable that the bacteria can withstand such temperatures if they are to have potential in heap leaching systems.

The growth rate constant of the isolates was assessed at different temperatures by incubation at 28°C, 37°C, 45°C and 50 °C in pyrite and yeast extract medium. The optimum growth temperature was then used for subsequent growth experiments and bioleaching experiments.

3.6.2 Growth rate constants of 4G, 5C and Cligga at different temperatures

It can be seen from Figure 3.12 that all three isolates grew optimally at 37°C as their exponential growth rate constants were higher at this temperature than the other temperatures tested. No growth was observed in any of the cultures at 50°C and no cells were detected by microscopy a few hours after inoculation, and the cell numbers were monitored for seven days with no growth at this temperature. The growth rate constants of the three isolates were slow at 45°C and growth at 28°C was only slightly lower than that at 37°C.

It can be clearly seen that isolates 4G, 5C and Cligga are mesophilic bacteria with temperature optima of 37°C although all grew to some extent up to a maximum of 45°C of those temperatures tested.

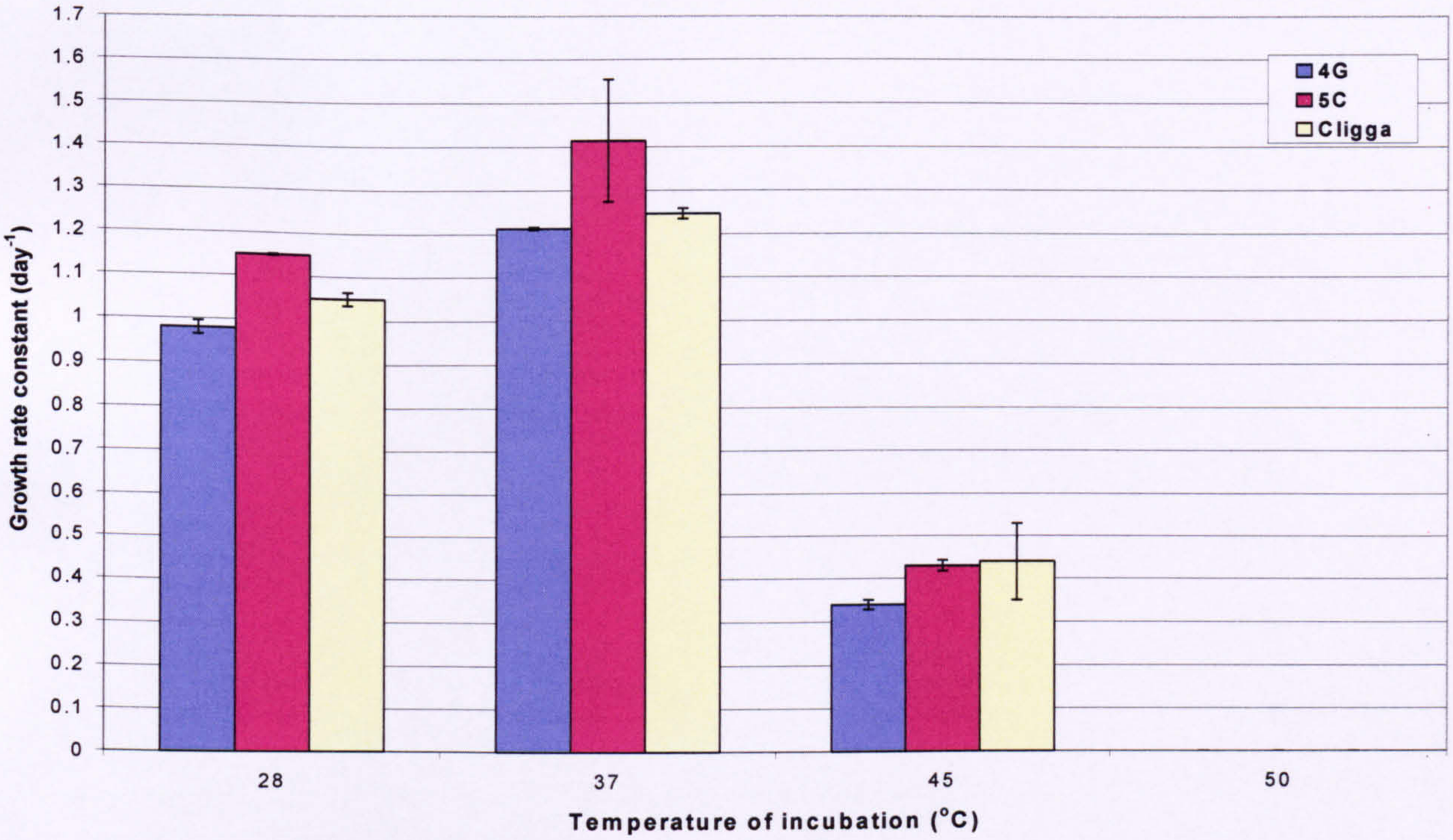


Figure 3.12 Growth rate constants of isolates 4G, 5C and Cligga in PSM at different incubation temperatures of 28°C, 37°C, 45°C and 50°C. Each datum bar represents the mean ± standard deviation of duplicate cultures.

3.7 Optimum pH for growth of isolated bacteria

3.7.1 Introduction

Acidophilic bacteria display optimum growth rate constants at a pH below 5.0, and many grow at or tolerate acidity as low as pH 0.5 (Johnson *et al*, 1992 and Johnson & Hallberg, 2003). There have been many reports of the effect of initial medium pH on the growth of iron-oxidising bacteria and it has been found that during growth of these acidophiles the pH is lowered due to the production of H_2SO_4 as a product of the metabolism of iron containing compounds. Seawater is an extremely efficient natural buffer and tends to stabilise any localised fluctuations in pH and therefore natural seawater and marine areas may not be ideal environments for the growth of bacteria that actually require low pH for viable growth. The normal pH range in seawater is from pH 7.2 to pH 8.2 with an average pH around pH 7.6 (Brown *et al*. 2002). However, localised low pH microenvironments may be formed in areas where chemical or biological processes increase the local hydrogen ion concentration.

Since the pH of the medium decreases during bacterial oxidation of iron, due to the production of H_2SO_4, it was decided to evaluate the effect of different low initial pHs on the growth rate constant of the three isolates. Media of pH 1.0, 1.5 and 2.0 were used, as pH 2.0 was the pH of the enrichment medium for the isolates and bioleaching processes are usually carried out in lixivant (leaching solution) of pH 2.0 or below. If these microorganisms are to be useful in biomining processes, it is important that they are able to grow in medium of pH 1.0 - 1.5 in order that they can tolerate the pH fluctuations during biooxidation.

3.7.2 Growth rate constants of strains 4G, 5C and Cligga at different initial pH

It can be seen from Figure 3.13 that strains 4G, 5C and Cligga grew optimally at pH 2.0 (the pH of isolation) but also grew slowly at pH 1.5 and pH 1.0. However, very low growth rate constants and long mean generation times were observed in medium at pH 1.0, and the generation times ranged from 38.22 hours to 57.14 hours. There was very little difference in growth rate constant and mean generation times in cultures at pH 2.0 and 1.5.

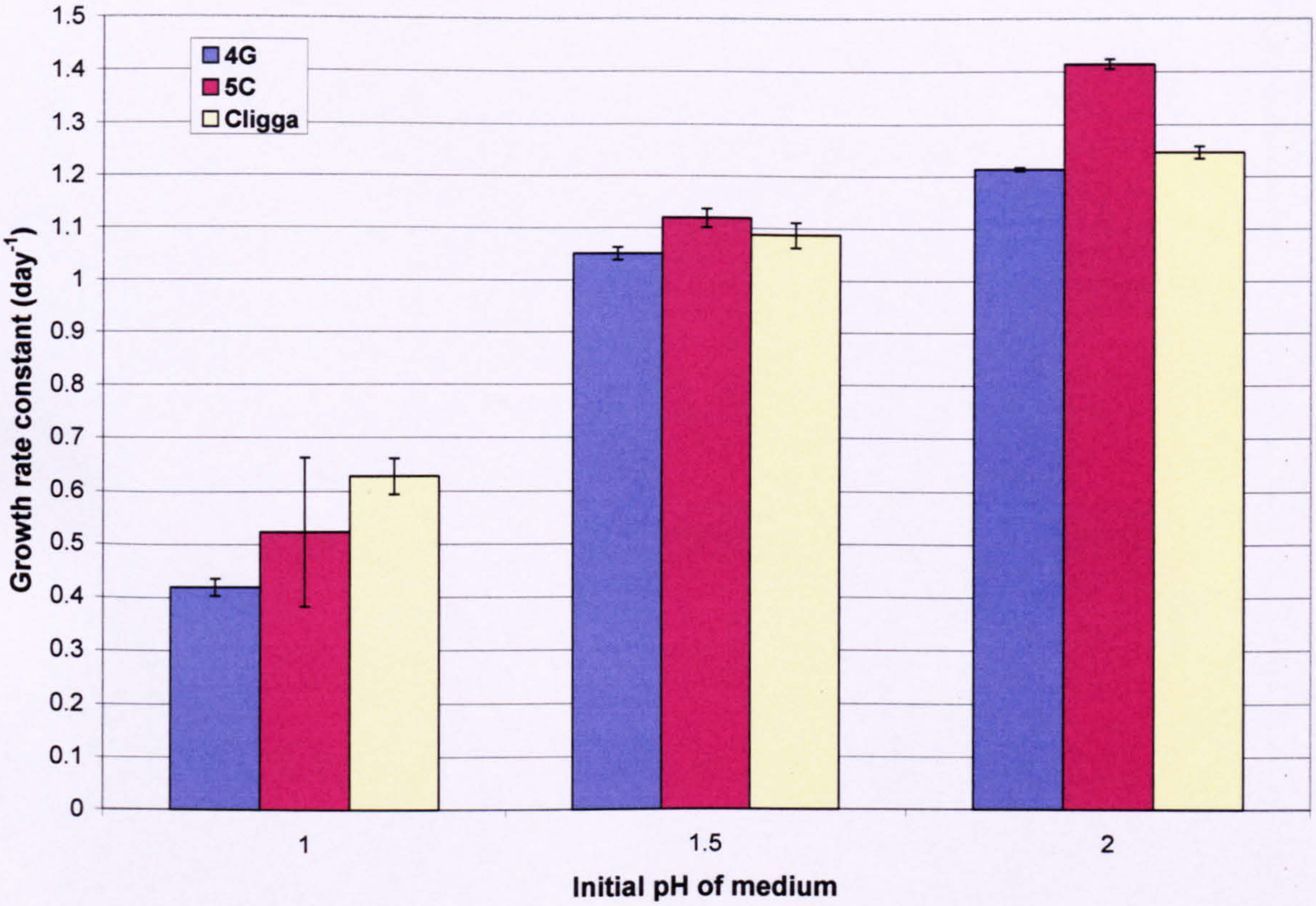


Figure 3.13 Growth rate constants of isolates 4G, 5C and Cligga in PSM medium of different initial pH; pH 1.0, pH 1.5, pH 2.0. Each datum bar represents the mean ± standard deviation of duplicate cultures.

3.8 Growth of the isolated bacteria on solid medium

3.8.1 Introduction

Acidophilic bacteria are notoriously difficult to culture on defined laboratory solid media (Das *et al*, 1989; Harrison, 1984; Johnson, 1995 and Bianchi *et al*, 1989). Many studies have sought to overcome the problems associated with growth on solid media, but few have been successful.

Successful attempts have included the overlay medium method developed by Johnson (1995). This method uses an underlay of medium solidified with agarose that has been inoculated with a culture of a heterotrophic acidophile (*Acidophilum sp.*) that has been found to be associated with iron and sulphur-oxidising bacteria in acid mine drainage environments and other high metal ion environs. An uninoculated layer of medium containing ferrous iron and/or tetrathionate is poured over the solidified bottom layer and allowed to solidify. The plates are incubated at 28°C for several days and then inoculated with the bacteria being studied. (Section 2.2.4 outlines the composition of overlay plates).

It is thought that the heterotrophic bacteria in the bottom layer of the plates utilise the polysaccharides in the top layer of the medium, thereby converting the potentially toxic complex organic compounds that could inhibit the growth of autotrophic bacteria to less toxic compounds (Johnson, 1995). This is the reason for incubating the plates for several days before inoculating with the test bacteria. Bacterial species such as *At. ferrooxidans* and *L. ferrooxidans* were successfully cultured on this medium and it was found by D. Barrie Johnson (University of North Wales, UK, personal communication, 2001) to be suitable for effective enumeration of these bacteria. However, to date, there is only one report in the literature of halotolerant, iron-oxidising acidophiles being grown on solid medium (Kamimura *et al*, 2001).

One problem that was encountered during this study was that many gelling agents do not solidify at the low pH required for growth of acidophiles. The low pH, combined with the high temperature of autoclaving lessen the chances of successful solidification of gelling agents and further complicates the development of efficient solid medium for maintenance and enumeration of iron-oxidising bacteria.

The following section describes attempts to grow the environmental isolates from this study on characterised medium for culture of acidophiles and details the development of a solid medium for maintenance of these strains.

3.8.2 Growth of the bacterial isolates on solid medium

Different types of solid medium were assessed for their ability to support the growth of the halotolerant isolates. The overlay medium developed by Johnson (1995) was assessed first without added salt but no growth was observed for any of the isolates that were either spread or streaked onto the plates. Sea salts (at a concentration of 10 gl^{-1}) were added to *Acidophilum sp.* liquid medium (Materials and Methods Section 2.2.11), in the hope that this terrestrial strain would become adapted to the increased salt concentration. The bacteria grew more slowly than usual, however, after the third subculture in this medium, *Acidophilum* sp. was used in the lower layer of the overlay plates. Sea salts (10 gl^{-1}) was added to the top layer of the overlay plates and then inoculated with the isolates and the plates were incubated at 37°C. However, no growth was observed by any of the isolates, even after prolonged incubation of four weeks. The isolates also failed to grow on TSM medium (Bianchi *et al*, 1989) which was assessed with 0, 10 gl^{-1}, 20 gl^{-1} and 30 gl^{-1} added sea salts.

It was possible that the high concentration of agarose in the plates inhibited the growth of the isolates and therefore alternative gelling agents that were thought to be less toxic to the bacteria were tested. Ferrous iron medium (see Materials and Methods section 2.2.3) was gelled using silica gel before ferrous sulphate and tetrathionate was added. However, even at high concentrations of silica gel (up to 30 gl^{1}) the medium did not solidify at pH 2.0, 3.0, 4.0 or 5.0. Carrageenan was also tested as a potential gelling agent again in ferrous iron medium, with addition of ferrous sulphate or tetrathionate as a potential substrate for bacterial growth. However, the medium did not gel at pH 2.0, 3.0, 4.0 and 5.0 even at high concentrations of carrageenan and therefore could not be used for culture of the isolated bacteria.

Finally, an attempt was made to develop a solid medium based on the liquid medium on which the isolates were routinely grown. Ferrous iron medium was used as a base

medium the addition of 8 gl^{-1} purified agarose (Molecular Biology Grade) was found to be sufficient to solidify the medium. The agarose solution was autoclaved separately at pH 7.0 so that the agarose did not hydrolyse during the high temperature of autoclaving and at the low pH. It was then cooled to the lowest point (below which it would solidify) and was then added to the autoclaved base medium (pH 2.0), the substrate (e.g. ferrous iron at a concentration 200 mM) added and the plates poured. The plates were not stacked during pouring as this was found to increase the cooling time and increases the chances of hydrolysis of the agarose, which would stop the plates from solidifying. (Materials and Methods Section 2.2.5 for composition of this medium). It was found that the medium with added sea salts needed extra agarose (an extra 2 gl^{-1}) in order to solidify.

Six variations of this medium was prepared including addition of:

- yeast extract (0.2 gl^{-1})
- yeast extract (0.2 gl^{-1}) and ferrous sulphate (200mM)
- ferrous sulphate (200mM)
- yeast extract (0.2 gl^{-1}) and sea salts (30 gl^{-1})
- yeast extract (0.2 gl^{-1}), ferrous sulphate (200 mM) and sea salts (30 gl^{-1})
- ferrous sulphate (200mM) and sea salts (30 gl^{-1})

Colonies were not observed on media without addition of sea salts and were only observed on medium containing yeast extract, ferrous sulphate and sea salts. These were raised white colonies which appeared in the first few days of incubation and these then turned rusty coloured after several days of incubation and in areas with a high density of colonies. All three isolates were able to grow when streaked on this medium and formed irregular shaped colonies when viewed using a dissecting microscope (see Figures 3.14 a-f. Cultures that were spread plated did not grow very well and therefore was not suitable for enumerating of the isolates by plate counting.

Figure 3.14a

Figure 3.14b

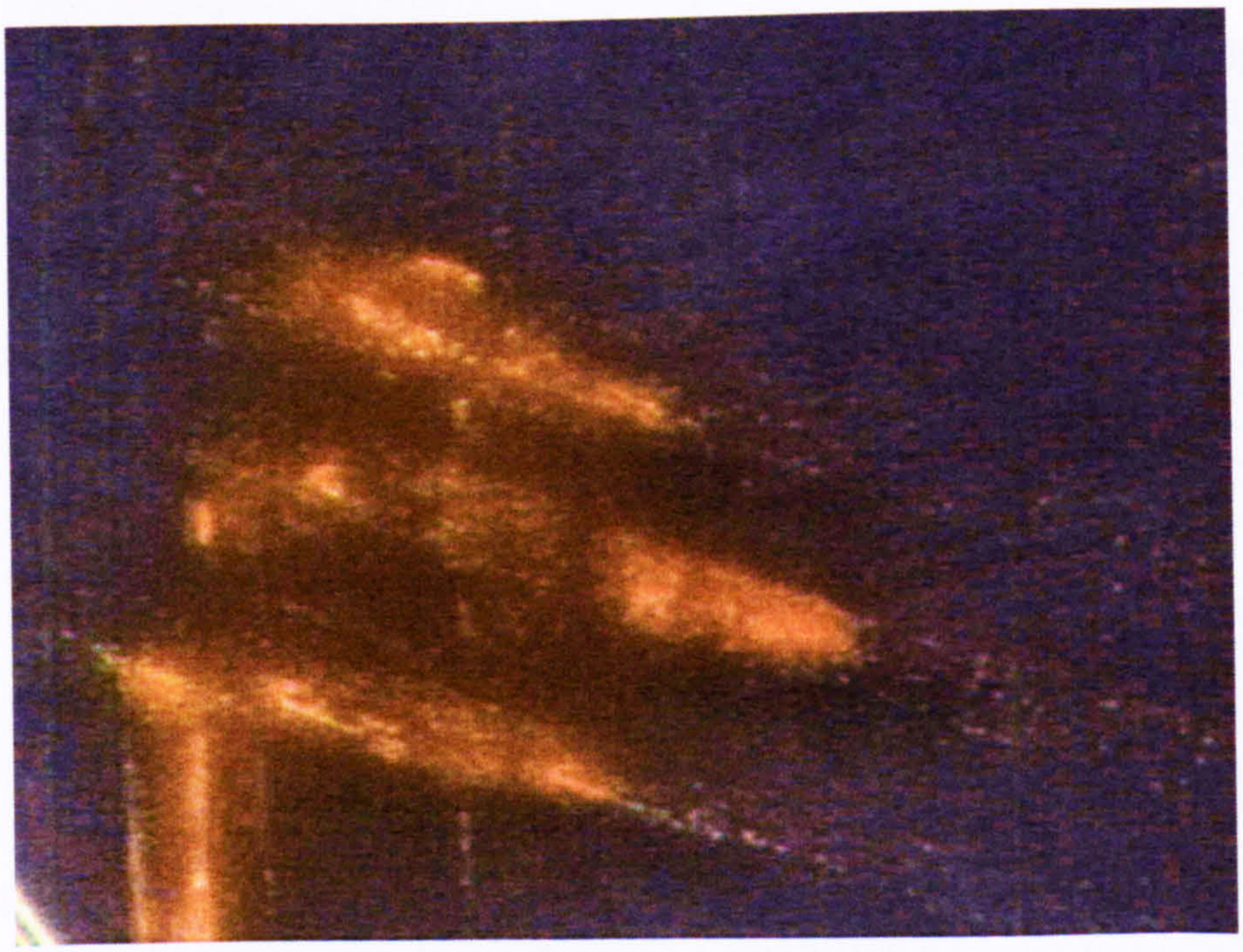


Figure 3.14c

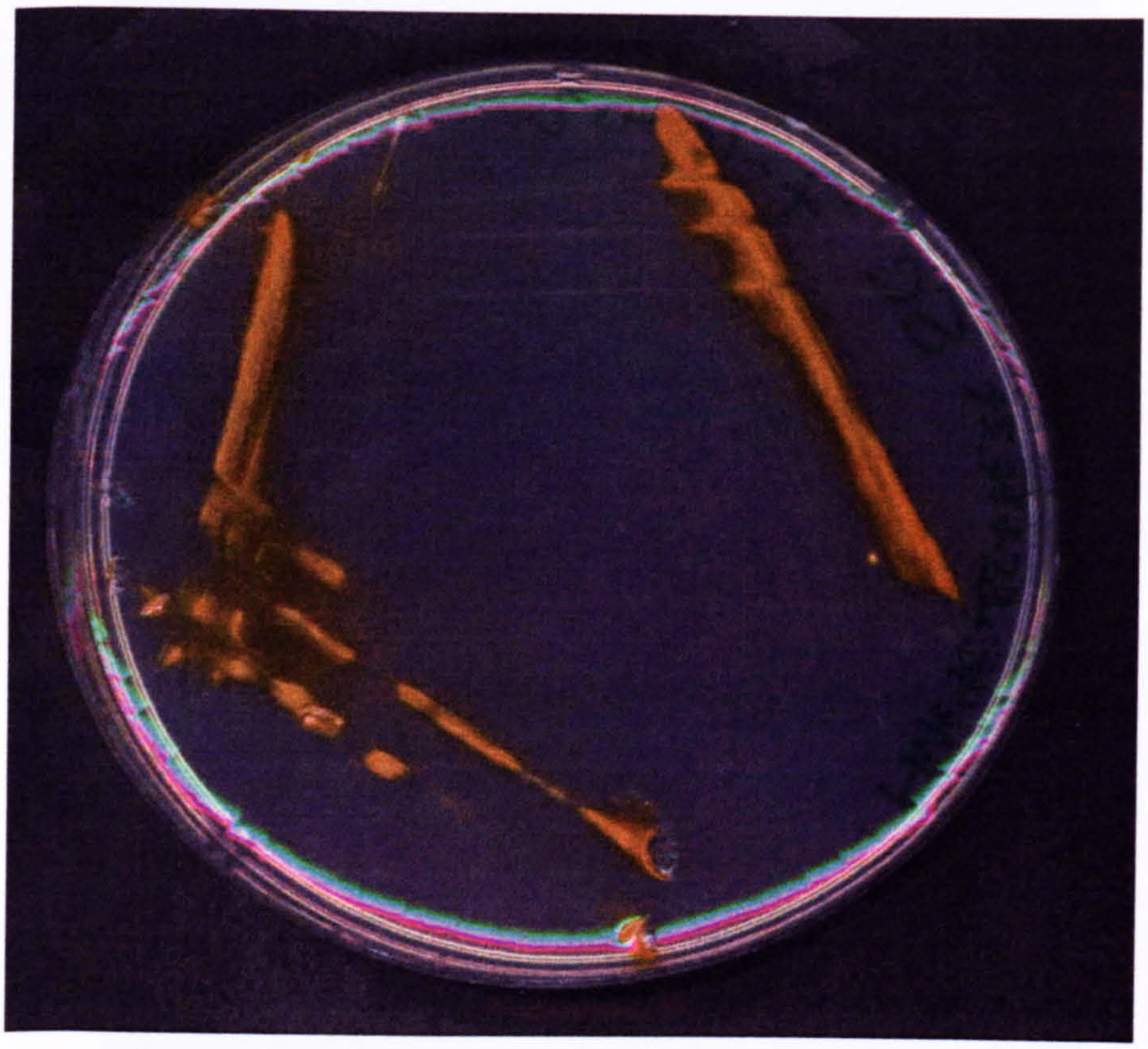


Figure 3.14d

Figure 3.14e

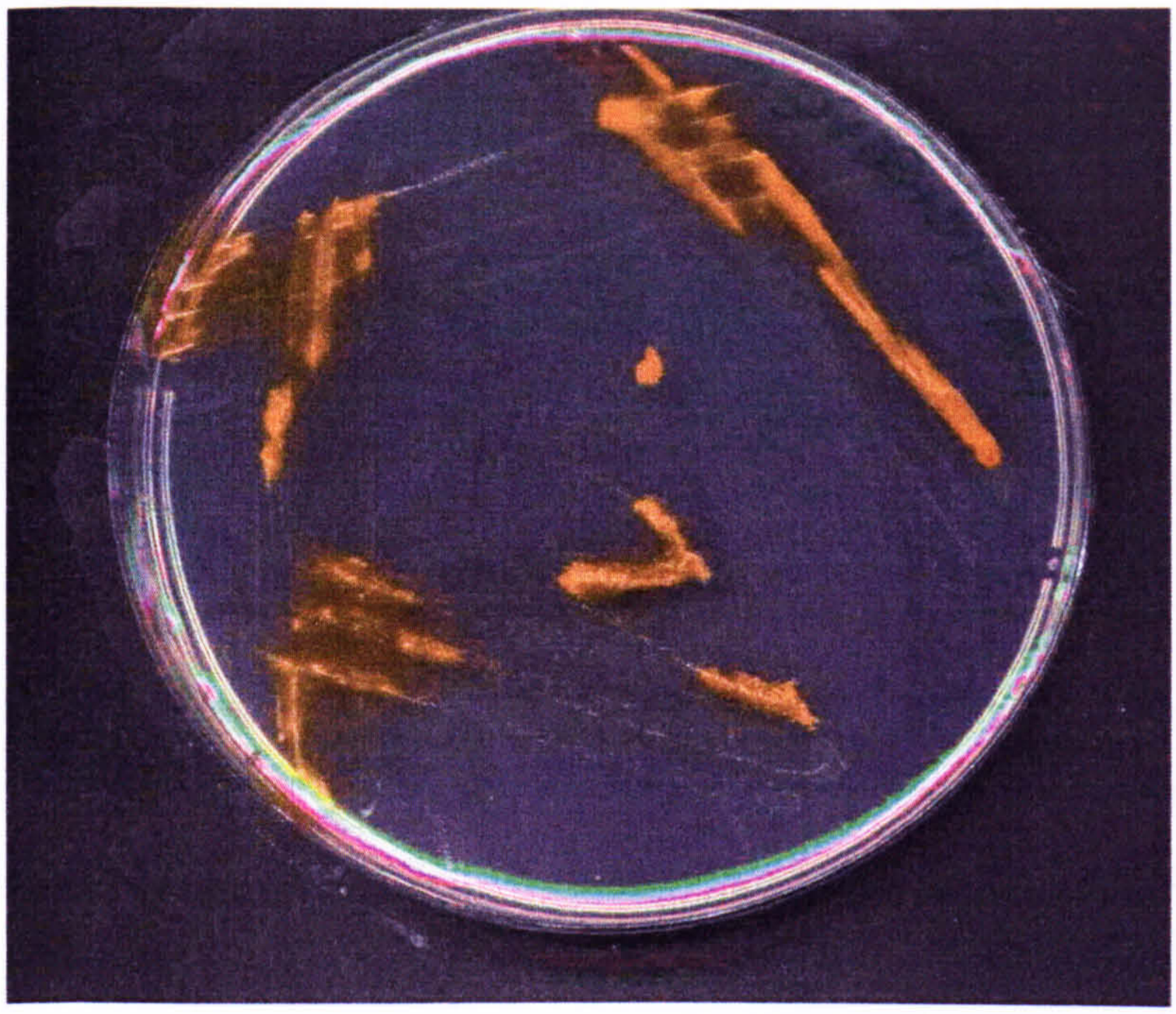
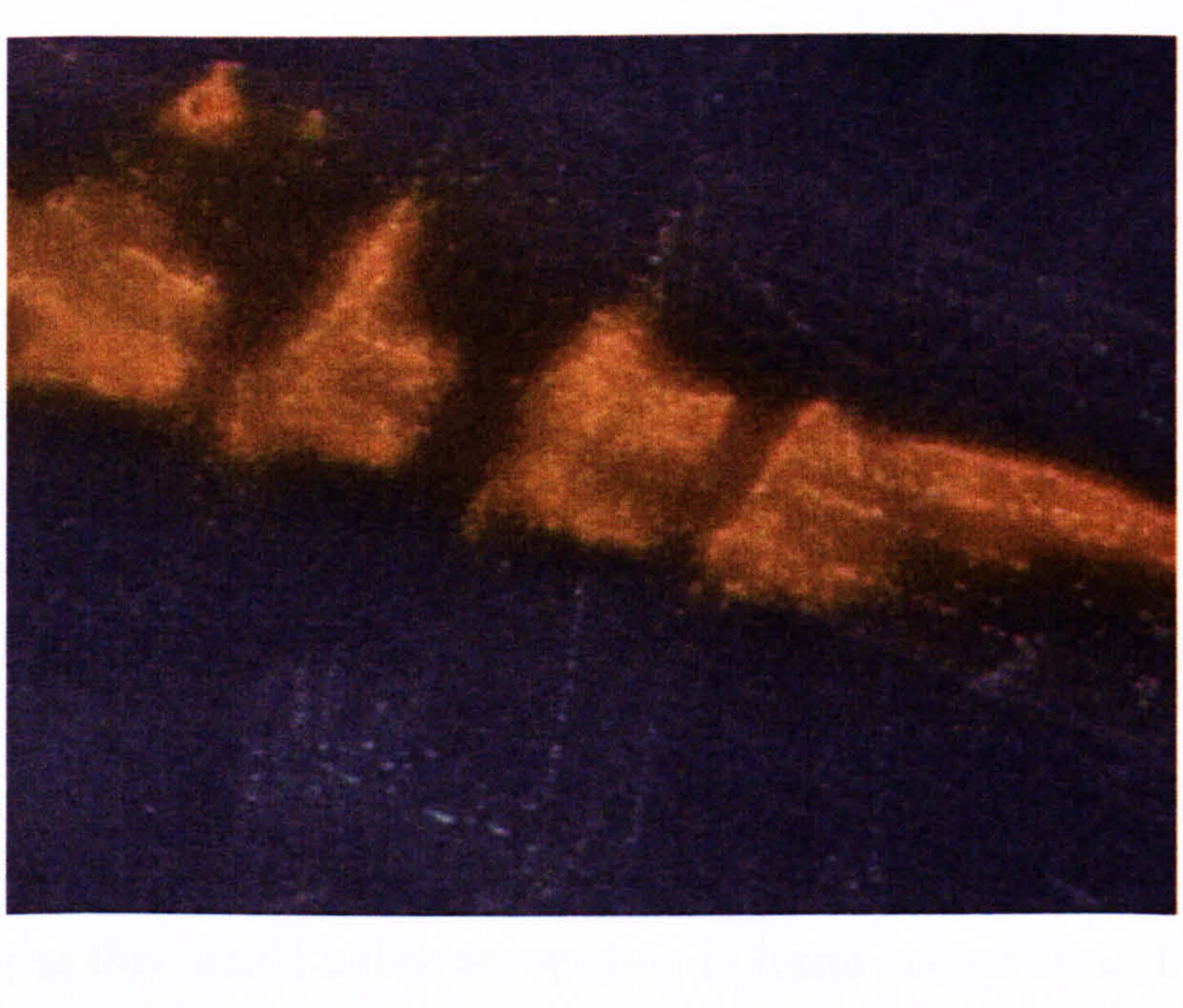


Figure 3.14f



Figures 3.14 a – f show growth of the three isolated bacteria on ferrous iron medium with yeast extract. Rusty coloured colonies can be seen on the surface of the plates in areas with a high density of colonies and white colonies in areas of less dense cell growth. Figures a and b shows growth of isolate 4G, Figures c. and d. show growth of 5C, and Figures e and f show the growth of isolate Cligga.

3.9 Growth of the isolated bacteria on ferrous iron, tetrathionate and elemental sulphur

3.9.1 Introduction

Acidophilic bacteria which use inorganic compounds as their electron source for energy production and growth utilise one or a variety of compounds including ferrous iron, elemental sulphur, reduced sulphur compounds such as pyrite and tetrathionate and a variety of iron and sulphidic mineral ores.

At. ferrooxidans can oxidise both ferrous iron and reduced sulphur compounds whereas *L. ferrooxidans* uses ferrous iron as its sole electron donor. Some members of the genus *Sulfobacillus* (such as *S. acidophilus*) can grow autotrophically on ferrous iron and elemental sulphur as well as heterotrophically on yeast extract (Norris *et al*, 1996).

Growth of the isolated bacteria on different substrates was assessed and is described in this section.

3.9.2 Growth of the bacteria on different substrates

The bacteria did not grow on ferrous iron saline liquid medium at pH 2.0 (see Materials and Methods section 2.2.3 for composition). Yeast extract was added to the ferrous iron saline medium (at a concentration of 0.2 gl^{-1}) but growth of the three isolates was not observed in this augmented medium by microscopy and there was no colour change of the medium. If ferrous iron were being oxidised to ferric iron (mediated by bacteria) the medium should change colour from a light green to a rusty orange colour.

Since all of the isolates mediate iron oxidation processes on solid ferrous iron medium it was expected that they would utilise ferrous iron in liquid culture also. Since some iron-oxidising bacteria require a reduced form of sulphur in order to grow on ferrous iron (Yahya & Johnson, 2002), tetrathionate was added to the ferrous iron saline medium but again growth was not observed. Powdered glass was added to the ferrous iron saline medium to provide a solid substratum for growth of the isolates, however, once again growth was not observed.

Pyrite saline medium (without pyrite) was also used as a base to test for growth of the isolates on tetrathionate and elemental sulphur. However, as with the previous medium growth of the isolates was not observed on either of these substrates, even when augmented with 0.2 gl^{-1} yeast extract.

3.10 Assessment of *T. prosperus* for use as a comparative benchmark microorganism for subsequent growth experiments with the isolated salt-tolerant bacteria.

3.10.1 Introduction

At the beginning of this study, *T. prosperus* was the only halotolerant iron-oxidising acidophilic, bacterial species that had been both characterised in the literature and was available in the culture collections. It was therefore decided to determine the growth properties of *T. prosperus* at different salt concentrations, with a view to using it as a benchmark organism for future physiology and bioleaching experiments using the isolated bacteria. The effects of salinity on the growth and the iron oxidation kinetics of *At. ferrooxidans* were also assessed and compared to those of *T. prosperus*, as a comparison of a terrestrial and marine bacterial species. This was a preliminary study and growth data are not presented.

During this study, monitoring of the growth of *T. prosperus* proved to be very difficult, due to the precipitation of intermediary products of iron-oxidation, such as jarosites, during culture on ferrous iron medium. Also, the cells tended to become attached to the solid particles when grown on ore substrates. Assessment of growth was made even more difficult due to the inability of *T. prosperus* to grow on solid substrates such as agar and agarose. Therefore, different enumeration methods were tested in order to overcome these challenges.

3.10.2 Assessment of enumeration methods for T. prosperus

Oxalic acid has previously been shown to help dissolve jarosites and release cells that may be trapped in clumps of jarosite (Ramsay *et al*, 1988; Schippers & Jorgensen, 2002). The effect of addition of oxalic acid was tested in order to dissolve jarosite and release *T. prosperus* cells that had become clumped with jarosite. However, it was found that even the addition of small amounts of oxalic acid caused the cells to lyse after a short period and therefore counting the cells in a haemocyctometer was not accurate as the number of intact and countable cells was decreasing throughout the counting period.

Prefilters were used to remove particulate iron precipitates and ore particles from the culture in order to facilitate accurate counting using a haemocytometer. However, it was found that in doing so, a large proportion of cells were removed by the filter as well, even after the sample was placed in a sonic water bath for 5 min before filtration. Therefore, subsequent enumeration was not accurate in determining total cell densities.

Protein determination methods were used on culture samples to measure biomass and to therefore monitor growth. However, it was found that 100 ml samples would have to have been used to be able to detect any protein (even when using micro detection method). It was found that even small amounts of iron in the sample interfered with the protein assay, even though samples were washed with H_2SO_4 before the assay. It was therefore decided that this was not a viable growth monitoring method.

Fluorescein diacetate dye was used to label the bacteria in order to enumerate cells under UV light using a haemocytometer. This was found to be an inaccurate method of enumeration because the gridlines on the haemocytometer could not be seen under UV and the light source was required to be switched between visible and UV. This therefore made it difficult to accurately monitor the number of cells in each square.

Samples of bacterial cultures were subjected to treatment in a sonic water bath, with a view to detaching the cells off any solid strata and therefore making any subsequent enumeration techniques determine a more accurate value for total cell densities in a culture. It was found that this treatment was successful and that 5 min in the sonic water bath was sufficient to detach cells. However, samples had to be counted immediately or the cells began to reattach to the solid particles, or to clump again.

3.10.3 Maintenance of T. prosperus

It was found that *T.prosperus* was best maintained by storing at room temperature. Cultures remained viable for up to 3 months, but were sub-cultured once a month to ensure good healthy growth of the sub-culture. Ferrous iron cultures were maintained by adding sterile pyrite to the flask when all of the ferrous iron had been oxidised. Stab cultures were prepared for this strain but when these were sub-cultured into liquid ferrous and pyrite medium growth was not observed. Growth of *T. prosperus* on solid

media was tested but it was found that this strain did not grow on any of the solid media types used in this study. *T. prosperus* could not be revived from freeze-dried cultures but only from elemental sulphur liquid cultures, ferrous iron cultures and ore cultures (DSMZ, personal communication).

3.10.4 Growth of T. prosperus and At. ferrooxidans at high salinity

Several different methods were attempted in order to improve the accuracy of growth monitoring and improve on the reproducibility of these methods. Ramsay *et al* (1988) reported that oxalic acid could be used to solubilise jarosite precipitates, however, this compound can lyse the cells and therefore the use of this compound was not an effective method for jarosite solubilisation in bacterial cultures. However, the use of a sonic water bath was found to release cells from the particulates for more effective enumeration and the samples were therefore subjected to this treatment before every cell count.

T. prosperus displayed optimal growth at 6 gl^{-1} NaCl (20% the salinity of seawater) and the highest iron-oxidation rate in medium with no salt source (data not shown). This strain was originally isolated from a geothermally heated marine environment (Huber & Stetter, 1989) and therefore would be expected to actually require higher salinity growth conditions for optimal growth. However, Kaye & Baross (2000) noted that hydrothermal fluids and water can range from one-tenth to greater than twice seawater (1-7% NaCl equivalent). These bacteria may inhabit areas of lower salinity within their marine environments and therefore do not exhibit optimal growth at high salinities. They may also survive in microenvironments with salt concentrations that are lower than those of the immediate environment.

The results of the effect of elevated NaCl concentrations on the growth of *At. ferrooxidans* concur with those of previous studies, which found that the growth of *At. ferrooxidans* is inhibited by high salt concentrations. It has been suggested that *At. ferrooxidans* does not have a chloride pump (Lawson et al, 1995) and therefore the membranes of this bacterium are susceptible to damage by influx of chloride ions.

It was found that *T. prosperus* was a difficult bacterial strain to maintain, and this fact had been noted by a number of other researchers (D. B. Johnson, University of Bangor, UK; D. Barr, Rio Tinto Technology Limited and Hans Hippe, DSMZ; personal communications). During this study it was found that *T. prosperus* cultures were best maintained by storing at room temperature with addition of sterile pyrite after all of the ferrous sulphate has been utilised by the bacteria. Many different solid media types were assessed for the growth of this bacterial strain, including overlay plates, TSM plates, and the nylon membrane culture technique. However, none of these solid media were sufficient to support the growth of *T. prosperus*. Since optimum growth was observed at 6 gl^{-1} for *T. prosperus* and 30 gl^{-1} for the isolated bacteria it was decided not to use this microorganism as a benchmark in subsequent experiments.

3.11 Discussion

Isolation of halotolerant iron-oxidising acidophilic bacteria has been attempted by many researchers, however, very few of these attempts have been successful. In this study, three halotolerant acidophilic strains were successfully isolated, maintained and their growth was assessed under a variety of conditions.

3.11.1 Enrichment of target bacteria

The medium that facilitated successful enrichment cultures was a pyrite saline medium (PSM) containing 30 gl^{-1} sea salts, 2 % (w/v) pyrite at a pH of 2.0 and incubated at 28°C. This enrichment medium satisfied all the conditions needed for growth of the target bacteria. Two of the isolates originated from sediment samples that were contaminated with mine tailings (waste from previous mining processes). These mine tailings usually comprise mostly of metallic pyritic minerals and so it was not surprising that pyrite was a suitable substrate, and utilised by these bacteria in the estuarine sediments.

Pyrite, as a source of iron and reduced sulphur may be the preferred source of substrate in marine sediments (of acidophilic, autotrophic bacteria) as iron occurs in the form of ferrous iron very rarely at the neutral pH and high oxidising conditions observed in seawater and marine areas. Any ferrous iron occurring in these environments is quickly converted to ferric hydroxide and other intermediate compounds of iron oxidation processes, leaving it in a form that is unavailable to iron-oxidising bacteria. It has been reported that some marine bacteria may reduce ferric to ferrous iron and thereby create a Fe^{2+} source that may possibly be utilised by iron-oxidising acidophiles (Lovely, 1991). However, this reduction would be minimal and unless it was happening in a localised area along with the growth of iron-oxidising bacteria, would not produce enough ferrous iron to provide a potential substrate for these bacteria. Reduction of ferric iron also forms a high pH during the reduction reaction and so would produce conditions that are not favourable to acidophilic microorganisms (Konhouser, 1997). Some ferrous iron is also produced by hydrothermal vent activity but again this may quickly be converted to an unusable substrate when it contacts oxygen and other reactive elements in the lower

temperatures of the sea around the vents. These reactions are then responsible for the formation of mineral precipitates that form the chimneys observed around such vents.

A halotolerant strain that grows optimally in medium with no added salt (*T. prosperus*) was isolated by liquid enrichment medium containing a mixture of mineral ores and was found to oxidise ferrous iron only very poorly and only when adapted to growth on ferrous sulphate (Huber & Stetter, 1989). Most of the other reports of the search for such halotolerant acidophilic bacteria have used ferrous iron (in the form of ferrous sulphate) in liquid enrichment medium as an electron donor for growth of target bacteria. Such studies include that of Kamimura (2001) who used $FeSO_4.7H_2O$ as a source of ferrous iron and used the formation of ferric hydroxide (reddish-rusty colour) as a positive indicator of the growth of iron-oxidising bacteria. The authors were looking for bacteria that could possibly be used in biohydrometallurgical processes and so by not using a mineral containing enrichment medium may have been selecting for bacteria that would grow on soluble iron but not mineral sources (Kamimura, 2001). Another example is Holden *et al* (1999), who used elemental sulphur and $FeSO_4.7H_2O$ in seawater medium despite the fact that the authors were looking for bacterial consortia which would be capable of bioleaching metals from harbour sediments and commercial mineral ore samples.

Thiosulphate enrichments did not produce any isolates during this study. Smith & Finazzo (1981) used thiosulphate enrichment medium and obtained a halotolerant iron-oxidising isolate (*T. intermedius*) from salt marsh sediments. However this strain was a neutrophile and had an optimum pH for growth around pH 6.0.

Enrichment medium with a salinity of 30 gl^{-1} sea salts proved to be a more successful enrichment medium than that with a sea salt concentration of 20 gl^{-1}. This result was explained when the effect of salinity on growth of the isolates was assessed (reported in Section 3.4). It was found that the exponential growth rate constants of the three isolates was higher during growth on 30 gl^{-1} sea salts than at 20 gl^{-1}, 10 gl^{-1} sea salts and with no salt added. Before the enrichment attempts in this study it was noted that previously isolated halotolerant iron-oxidising bacteria usually had salinity optima that were below that of the natural environment of the source sample (Smith & Finazzo, 1981 and Huber & Stetter, 1989). Therefore, it was remarkable that the enrichment

medium with 30 gl^{-1} sea salts resulted in the isolation of the halotolerant bacteria described in this study.

Successful colony formation by iron-oxidising bacteria was observed using a nylon membrane culture method (developed by L. Yan, PhD Thesis, Heriot-Watt University, 2001). The liquid medium under the membrane contained minimal medium with ferrous sulphate, and 30 gl^{-1} sea salts at a pH of 2.0. However, these isolated bacteria were unable to grow in the same medium, in liquid culture without the membranes or in pyrite or thiosulphate media (with 30 gl^{-1} sea salts, pH 2.0). The nylon membrane used should allow dissolved chemical species to move freely through it. However, it is thought that the membrane could also offer some protection from the salt because of the production of exopolysaccharides by the bacteria in contact with a solid surface and subsequent formation of protective biofilms. Therefore bacteria, which may have a lower tolerance to salt, may be culturable using this method even in high salt medium. This might explain why these microorganisms (Calenick and Tressillian enrichments) were unable to grow on sub-culture into liquid salts medium, as there is no available solid strata for the formation of biofilms.

It was also found that the nylon membrane was not an ideal method of growth of the target bacteria due to the very high rate of evaporation of the medium and subsequent drying of the membrane, coupled with the very long incubation times needed for culture of the bacteria.

3.11.2 Growth of the bacterial isolates at different concentrations of sea salts

The majority of the characterised halotolerant iron-oxidising bacteria reported in the literature may tolerate elevated levels of salt, but many have salinity optima that are well below their maximum tolerable level or that of their natural environment. For example, Holden *et al* (1999) isolated metal mobilising bacteria from seawater with a concentration of 3% (w/v) NaCl, but their strains were found to grow only between 1% and 2% NaCl.

An iron-oxidising neutrophile (*T. intermedius*) was isolated from a salt marsh with an interstitial water salinity of 3% NaCl, however, the optimum salinity for growth of this

strain was only 1% NaCl, one third of that of the natural environment (Smith & Finazzo, 1981). These observations led Smith and Finazzo to believe that some bacteria grow at their optimal rate only rarely in nature. The growth of marine iron-oxidising isolates has been only rarely assessed at different salinities, but is an important parameter in order to investigate the salinity optima of this type of bacteria.

The growth of the isolated bacteria on medium with different concentrations of sea salts was reported in Section 3.4. It was found that (contrary to many reports on bacteria isolated from similar environments) growth rate constants actually increased with increasing salt concentration in the medium. This was a highly desirable result as it was hoped that these bacteria might have potential in high salinity bioleaching operations with a high availability of seawater for use in the process. The growth rate constants of all three isolates were highest in medium with 30 gl^{-1} added sea salts and progressively decreased in medium with lower salinities. This provides evidence that these bacteria are highly adapted to their natural environments, as all were isolated from environs ranging from 3.2 % to 4.2 % salinity. This is the first report of halotolerant iron-oxidising bacterial species having salinity optima that are equivalent to those of the environment from which they were isolated.

It was noted that many reports of the isolation of halotolerant iron-oxidising bacteria from marine environments used sodium chloride as a direct salt source in enrichment cultures and subsequent assessment of growth responses (Smith & Finazzo, 1981 and Huber & Stetter, 1989). Only two studies have used seawater medium to isolate this type of bacteria (Tilton *et al*, 1967a and Holden *et al*, 1999).

Bacteria are usually highly adapted to their natural environment and therefore, the importance of mimicking these environmental conditions during isolation of specific types of bacteria cannot be underestimated. It seems remarkable that most of the reports of isolation of the target bacteria did not use seawater (either natural or artificial). This may be why very few of these reported isolation attempts or maintenance of isolates have been successful and shows that the use of sea salts is much more conducive to the isolation of halotolerant bacteria than NaCl alone.

Each of the three isolates exhibited higher growth rate constants when grown in medium containing 30 gl^{-1} sea salts rather than 30 gl^{-1} NaCl or in the absence of salt.

These results suggests that there may be a component present in the sea salts, which promotes the growth of these isolated bacteria. The exact composition of the sea salt mixture is not defined (details were not available form Sigma), but as it comes from natural seawater it is assumed to be close in composition to that of average open seawater. There is a slightly lower concentration of NaCl in the sea salt medium as in the NaCl medium, which may explain the difference in the growth rate constants between the two media (i.e. less Cl^- toxicity). However, this fact alone cannot explain the extent of the differences in the observed growth rate constants. Isolate 4G has a growth rate constant in sea salts medium that is 2.5 times that observed in NaCl medium and isolate 5C has a growth rate constant in sea salts medium that is 4.1 times that in NaCl medium. Also, isolate Cligga has a growth rate constant in sea salts medium that is 3.8 times higher than when this strain is grown in NaCl medium.

Chloride and sodium are the two most abundant ions in seawater, however SO_4^{2-}, Mg^{2+}, Ca^{2+}, K^+, HCO^{3-}, Br^-, H_2BO^{3-} and Sr^{2+} combine with these to make up 99.9 % of the dissolved constituents of seawater (Brown et al, 2002). Therefore these ions and other trace elements may actually be required for the optimum growth of the bacteria that are isolated from marine sources, such as those in this study.

The question of what actually constitutes a truly marine bacterial species is a highly debated one. It is not sufficient that a bacterial strain is isolated from the marine environment and that it grows optimally at the salinity of that environment. A common definition of marine bacteria is that coined by Macleod, that states that "marine bacteria are those that have a obligate requirement for Na^+ above what is available at background levels" (Macleod, 1965). There are so many different conditions, chemical, biological and physical, observed in marine environs that it is unlikely that a single definition could encompass them all, and therefore this topic will surely continue to be a highly contested issue.

There are many gaps in Macleod's definition including that fact that many bacteria isolated from environments which would not usually be classed as marine (such as salt marshes and hypersaline lakes), have an obligate requirement for Na^+. This includes *T. halophilus*, isolated from a hypersaline lake, which has a NaCl optimum of 0.8 –1.0 M and, while it will not grow below this level, tolerates NaCl levels up to 4 M (Wood & Kelly, 1991).

The results reported in Section 3.4 demonstrate that the bacteria isolated in this study do not exhibit an obligate requirement for Na^+ as they grow in medium with no added salt source, albeit at a greatly reduced rate. According to Macloed's definition, it therefore follows that these bacteria cannot be termed as 'marine bacteria' despite having been isolated from marine environments. Nonetheless, the results show that the three isolates have higher exponential growth rate constants, lower mean generation times, and higher final cell yields when grown in the presence of 30 gl^{-1} sea salts rather than the same concentration of NaCl or with no added salt. Therefore, this salt source and concentration was used for subsequent growth experiments and high salinity bioleaching studies (Chapter 5).

3.11.3 Comparison of the growth of the isolated halotolerant bacteria under mixotrophic and heterotrophic conditions

Some iron and sulphur oxidising bacteria exhibit higher growth rate constants when their medium is augmented with an organic carbon source, such as yeast extract. Such bacteria include *Sulfobacillus thermosulfidooxidans* and *Sb. acidophilus* (Norris *et al*, 1996). *Sulfobacillus sp.* are capable of autotrophic growth (on inorganic substrates), heterotrophic growth (on organic carbon sources) or growth on both types of substrate during mixotrophic growth. Chemoautotrophic fixation of CO_2 requires large amounts of CO_2 to be fixed in order to meet the demands for carbon for growth of cells. Therefore, in cultures that are not sparged with CO_2 or air, carbon may be a growth rate constant -limiting chemical. It is therefore beneficial to provide a carbon source to facilitate the production of higher cell biomass.

Very long lag phases of up to 4 days were observed in heterotrophic cultures of both isolates 4G and Cligga, before growth was observed (Section 3.5). In these cultures, lag phases were shorter under mixotrophic growth conditions but ultimately, the exponential growth rate constants were higher in heterotrophic growth mode. Strain 5C exhibited a lag period of two days under mixotrophic conditions whereas exponential growth was reached very quickly by this strain in heterotrophic conditions. The overall exponential growth rate constant of 5C in heterotrophic medium was over twice that observed in mixotrophic medium.

During heterotrophic growth, the mean generation times of 4G, 5C and Cligga were 7.0 hours, 6.8 hours and 7.7 hours respectively (Section 3.5.5). These were extremely short generation times as compared to mixotrophic growth which were 19.8 hours, 16.7 hours and 19.3 hours respectively. This high growth rate in heterotrophic medium is presumably due to the higher availability of a carbon source in the form of yeast extract and negates that need for high levels of atmospheric CO_2 fixation, thereby directing more energy into cell metabolism and growth. The long lag periods observed in cultures of 4G and Cligga may be due to the time needed for the production of proteins required to switch to oxidation of organic carbon sources for metabolism.

High amounts of inorganic substrates need to be utilised during autotrophic and mixotrophic growth in order for quantifiable growth of this type of bacteria. The oxidation of these substrates produces less energy than that produced by heterotrophic growth. Therefore, growth rate constants will be slower, mean generation times longer and final cell biomass lower, as was observed in Section 3.5.

3.11.4 Effect of temperature on growth of the bacteria

Optimum temperatures for the growth of bacteria can vary greatly from those of the environments from which they was isolated, and it is therefore important to assess optimum growth temperatures to ensure that investigations are carried out at the optimum growth rate constant for the conditions.

Many previously characterised iron-oxidising bacterial strains are thermophilic or hyperthermophilic, however most of the halotolerant iron-oxidising species are mesophilic and have optimum growth temperatures between 30°C and 37°C (e.g. *T. prosperus*, Huber & Stetter, 1989; Strains KU2-11 and SA, Kamimura *et al*, 2001, 2003).

Most *Sulfobacillus sp.* described in the literature are thermophiles e.g. *Sb. thermosulfidooxidans* T_{opt} 80°C; *Sb. acidophilus* T_{opt} 45°C; and *Sb. disulfidooxidans* T_{opt} 50°C (Dufresne *et al*, 1996; Karavajko *et al*, 1990 and Norris *et al*, 1996). However, there have recently been reports of novel mesophilic gram-positive bacteria which may be closely related to *Sulfobacillus sp.* (Yahya & Johnson, 2002). These strains

(*Sulfobacillus* L-15 and Riv-14) have a optimum growth temperature of 35°C. As the strains isolated in this study have been found to be similar to *Sulfobacillus sp.* growth at a variety of temperatures was assessed; 28°C, 37°C, 45°C and 50°C.

It was found that all three bacteria isolated grew optimally at 37°C as their exponential growth rate constants were higher at this temperature than the other temperatures tested (see Section 3.6). No growth was observed in any of the cultures at 50°C, and no cells were detected a few hours after inoculation and the cell numbers were monitored for a further 7 days with no obvious signs of growth being observed. The growth rate constants of the three isolates were lowest at 45°C and growth at 28°C was only slightly lower than that at 37°C.

It was concluded that all three bacterial strains were mesophilic but could grow at temperatures up to a maximum of 45°C of those temperatures tested. As a result growth response and bioleaching experiments involving these strains were carried out at an incubation temperature of 37°C.

3.11.5 Effect of initial medium pH on growth of the isolated bacteria

Acidophilic bacteria are considered to be those with pH optima below pH 5.0, extreme acidophiles have pH optima below pH 3.0. and many of the previously characterised halotolerant iron-oxidising bacteria have pH optima around pH 2-3. However, there are some exceptions, such as the iron-oxidising bacteria *T. intermedius*, a neutrophile, that has a pH optimum of 6 and *T. halophilus,* with pH optima of 7-7.3 (Smith & Finazzo, 1981 and Wood & Kelly, 1991).

The strains described in this study were isolated in medium of pH 2.0 and it was hoped that they could be used in bioleaching of metallic ore processes which preferentially takes place at pH 2.0 and below. The growth of the strains was therefore assessed at pH 2.0, pH 1.5 and pH 1.0 to see whether this extreme acidity could be tolerated.

It was found that strains 4G, 5C and Cligga grew optimally at pH 2.0 (the pH at which they were isolated) but also grew at pH 1.5 and pH 1.0. Very low growth rate constants and long mean generation times were observed were observed in medium at pH 1.0,

when the generation times ranged from 38.22 hours to 57.14 hours. There was very little difference in growth rate constant and mean generation times in cultures at pH 2.0 and 1.5. This was as expected as the pH decreased during growth of these acidophiles via the production of large quantities of H_2SO_4 as a product of metabolism. During some bioleaching experiments in this study, the pH was found to decrease to pH 1.5 from an initial pH of 2.0 during biooxidation of metallic ores (see Chapter 5). These isolates would survive and grow under the low acidity of biooxidation processes.

Some questions to which the answers remains elusive are; do these bacteria grow at this low pH in their natural environment and if so where are these low pH locales found in marine areas and how are they maintained at this low pH? As has been noted, seawater is a highly efficient natural buffer and tends to stabilise any local fluctuations in pH, which make these questions even more interesting.

During preliminary investigations prior to this study, it was found that *T. prosperus* could withstand a lower pH than normal when grown on mineral ore as compared to growth on ferrous iron (data not shown). This led to the hypothesis that solid sub-strata may provide a protective effect from extremely low pH. There have been many reports on the attachment of autotrophic bacteria to mineral ore particles via the production of exopolysaccharides (Costerton *et al*, 1978; Escobar *et al*, 1996; Porro *et al*, 1997: Sampson and Blake, 1999; Sampson *et al*, 2000 and Shrihari *et al*, 1991). The biofilms, thus formed, may provide protective micro-environments where the localised low pH formed from production of H_2SO_4 might be maintained and protected from the buffering effects of open seawater, in marine sediments, marine iron-containing structures and rock surfaces. Further investigation of the protective role of slime layers against external physical and chemical stresses would prove to be very interesting.

3.11.6 Growth of the isolated bacteria on solid medium and other substrates

As previously noted, it is notoriously difficult to grow iron-oxidising acidophilic bacteria on solid medium. This is due in part, to the low pH need to facilitate growth of these acidophiles. Traditional gelling agents such as agars hydrolyse easily under low acid conditions and are not suitable for growth of acidophiles. Many of these types of

bacteria are sensitive to high levels of complex organic substrates such as agar and so such gelling agents in solid medium may well be toxic to these bacteria.

Some researchers have developed media types, which have been utilised in the successful culture of terrestrial acidophilic strains, such as the heterotrophic bacteria overlay culture method developed by Johnson (1995). However, such methods do not translate successfully to the culture of halotolerant bacteria, due to the toxicity of the NaCl to the heterotrophic bacteria in the bottom layer. It was also found in this study, that medium with high salt levels needed extra gelling agent for solidification, since the elevated salt concentration encourages the hydrolysis of the gelling agents.

The isolated halotolerant bacterial strains were found to form rust coloured, irregular shaped colonies, when grown on medium gelled with high purity agarose, containing ferrous sulphate, yeast extract and sea salts. It is interesting to note that although the three isolates do not have an absolute requirement for NaCl or sea salts in liquid medium, attempts to culture them on solid medium with no added salt source were unsuccessful. Similarly, these isolates exhibit good growth rate constants in heterotrophic liquid medium with no added ferrous iron source but were found not to grow on solid medium without an added iron source (in the form of ferrous sulphate).

Some studies have reported the difference in growth of bacterial strains when grown in liquid medium as compared to growth in solid medium. These studies have subsequently noted the difference in metabolic functions and the involvement of the initiation of expression of different genes under different conditions (Jensen & Fenical, 1994 and Prigent-Combaret *et al*, 1999). The lack of growth of the isolated strains on heterotrophic medium without iron might be due to the inefficient expression of the genes needed to switch from planktonic growth to growth on solid substrata, which may be dependent on the presence of an iron source.

The lack of growth on heterotrophic solid medium may be due to the decreased availability of the organic carbon source in solid medium. For example, Meargy (1995) noted that during metal resistance studies on liquid and solid medium, dissolved constituents in solid media form complexes with the gelling agents that reduce the bioavailability of these compounds for the growth of inoculated bacteria. Therefore, measured resistance to metals was found to be higher on solid medium than in liquid

medium. This may help to explain the lack of growth on solid heterotrophic medium. The strains may actually require a certain level of iron in the medium, which may be present in heterotrophic liquid medium as impurities. However, as suggested, by Meargy (1995), these levels may not be sufficiently available for adequate growth of the isolates on solid medium.

The fact that the isolated bacteria did not grow on ferrous iron, elemental sulphur or tetrathionate was not wholly unexpected. This is likely to be due to the fact that these elements and compounds rarely exist in their bioavailable form in marine areas and seawater, and so would not be a natural substrate of the bacterial strains *in situ*. When these substrates do occur in marine areas, they are usually formed by biological conversions. Therefore, the acidophilic bacteria would need to be living in microenvironments where these compounds where being formed by the metabolism of other types of organisms, in order to be able to utilise sufficient amounts of these substrates.

The isolated strains can be termed as iron-oxidising because they have been grown in pure culture with only pyrite as an available substrate. Even though they do not oxidise ferrous iron in liquid culture they produce rust coloured colonies on solid ferrous iron medium. This colour production is indicative of the conversion of ferrous to ferric iron, which will not happen abiotically at the low pH of the solid medium (pH 2).

In summary:

Three strains of iron-oxidising, acidophilic, halotolerant bacteria were isolated from marine areas known to be high in iron containing compounds. The isolated bacteria were all gram-positive, spore-forming rods that grew on pyritic minerals, yeast extract and combinations of these substrates. The bacteria did not have an obligate requirement for NaCl but had higher growth rate constants when grown on medium with an added salt source, and preferentially on sea salts as opposed to NaCl. Higher growth rate constants were observed when the bacteria were grown on heterotrophic medium than on mixotrophic medium. All the strains grew optimally at 37°C but could tolerate growth temperatures up to 45°C. The isolates all had an optimum growth pH of 2.0, but tolerated media of pH 1.5 and pH 1.0. The strains had an optimum salinity for growth of 30 gl^{-1} sea salts and growth rate increased with increasing salinity from 0 – 30 gl^{-1}. Growth was not observed in liquid medium containing ferrous sulphate, elemental sulphur and tetrathionate but the bacterial isolates were found to form rust coloured colonies during culture on solid agarose medium containing ferrous iron, sea salts and yeast extract

Chapter Four

Phylogenetic characterisation of halotolerant, acidophilic bacteria isolated from sample sites

Chapter Four: Results

4.1 Introduction

Acidophilic, iron-oxidising microorganisms belong to three distinct groups. The main ones are the gram-negative rod-shaped bacteria (α,β,γ-proteobacteria), the low G+C gram-positive bacteria and the group belonging to the Kingdom Archaea. These microorganims are phylogenetically diverse and belong to different evolutionary lineages. However, they are found to live co-operatively, in very similar environments and are thought to complement each other's growth by the production of different products from their physiological processes that can be then utilised by other acidophiles. The range of environments exploited by these microorganisms include mine sites, areas of acid mine drainage contamination, coal spoil heaps, geothermal sites and other environments characterised by low acidity and a high metal content.

The full extent of the diversity of sulphur and iron-oxidisers from these environments is probably not yet realised due to culture and maintenance inadequacies (Lane *et al*, 1985 & 1992). It is however, being rapidly elucidated due to development of molecular methods such as denaturing gradient gel electrophoresis (DGGE) and improvements in in-situ molecular ecology methods. (Amann *et al*, 1992; Liesack & Stackebrandt, 1992 and Nicolaisen & Ramsing, 2002)

In this study, DNA was extracted from pure cultures of the isolated halotolerant bacteria and universal primers (9F and 1542R, Lane *et al*, 1985) were used to amplify a large portion of the 16S rDNA region. Internal primers were then used to sequence smaller portions from the large amplified region. (the primers used were 27F, 342F, 685F, 785F, 1099F, 524R, 802R, 1115R, 1492R). See Materials and Methods section 2.7.

The obtained sequences were then aligned using the program CAP EST ASSEMBLER (FIRC Institute of Molecular Oncology, Italy – http://bio.ifom-firc.it/ASSEMBLY/assemble.html) and searches for similar sequences were performed using NCMB BLAST software http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al*, 1997). Bacteria with high levels of sequence identity were noted, and a similarity matrix was constructed for the isolated bacteria and their closest relatives using the sequence identity values obtained. Clustal W (CMBI, Centre for Molecular

and Biomolecular Informatics-http://www.cmbi.kun.nl/) was then used to align the obtained 16S rDNA sequences with those of characterised related bacteria and to calculate nucleotide substitution rates. This alignment data in PHYLIP format was then fed into TREEVIEW (Page, 1996) to construct phylogenetic trees. Frequently, the differences in 16S rDNA between closely related strains are usually concentrated in one or two hypervariable regions and sequence analysis should take this into consideration (Stakebrandt & Rainey, 1995). Therefore the identities and construction of trees was performed for both the hypervariable region (first 600 base pairs) and also with longer sections of 1200 base pairs of the 16S rDNA region obtained.

4.2 16S rDNA sequences of the three isolated bacterial strains

4.2.1 Introduction

As divergence of the primary lines of bacteria descent occurred during early biotic history, highly conserved molecular chronometers (such as those represented by 16S rRNA) are required for accurate bacterial phylogenetics (Woese, 1987). Molecules of rRNA also contain sufficient variability so that relationships between closely and distantly related groups can be determined (Devereux & Willis, 1995). Therefore, the partial nucleotide sequences of the 16S rDNA region of the three isolated strains of halotolerant, acidophilic, iron-oxidising bacteria were determined and are illustrated in Figs 4.1, 4.2 and 4.3). The 16S rRNA sequences of the three strains have been deposited in the EMBL/Genbank database under accession numbers 4G – AY371272; 5C – AY371273; Cligga – AY371274. These sequences were also checked for Chimeras using the Ribosomal Database Project software (Cole *et al*, 2003).

4.2.2 Sequences of 16S rRNA gene from isolated bacterial strains, 4G, 5C and Cligga

1 TATTTATCTGGTAAGGGCACGTGCGGCGCTCCTAAACATCAATCCAACGG
51 CGCCTTCGGGCGCAGCGGCGGACGGGTAGGAACACGTGACGTAACCGGGC
101 GTCCGGTGGGGCATATCGGGCCGAAAGGCGCGGCAATCCCGCATAGCGTC
151 CCCGCGTGCGCAAGCAAGGCGGGGGAAAGGCCTTCGGGTCGCCGGATGGG
201 GGGCTCGCGGCGCCATTAGCTAGTTGGGGGGGTAACGGCCTCCCAAGGCG
251 ACGATGGGTAGCCGGCCTGAGACGGGTGATCGGCCACACTGGGACTGAGA
301 CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGCGGAATCTTCCACAATG
351 GGCGCAAGCCTGATGGAGCAACCGCCGCGTGAGTGAAGACGGCCTTCGGG
401 TTGTAAAGCTCTGTCTGTCGGGACGAGGACCCGGGTGCAAACCCGGGGGG
451 GACGGTACCGGCGGAGGAAGCCCCTGCAAACTACGTGCCAGCAGCCGCGG
501 TAAGACGTAGGGGGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGGGCGT
551 GTAGGCGGGTATCACACTGTAGCAGGTTTTCAGCCGTCGGCTCACCCGAC
601 GGAGGGCGGCTAAACGGTGGCACTTGGAGGGCAGGAGAGGTGCACGGAAT
651 TCCTGGTGGAGCGGTGAAATGCGTAGAGATCAGGAAGAACACCCGTGGCG
701 AAAGGCGGTGCACTTGGCCTGACCCTGACGCTGAGGCGCGACAGCGTGGG
751 GAGCGAAACGGATTAGATACCCCGGTAGTCCACGCCGTAAACGATGGATA
801 CTAGGTGTCGCGGGGGTCCACCCGGCGGTGCCGGAGCTAACGCACTAAGT
851 ATCCCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACG
901 GGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGACGCAACGCGCA
951 GAACCTTACCAGGACTAGACGGGATCGTGAGCGCCGCGAAAGCGGCGGCC
1001 TCTTCGGAGGAGCGGTCGTCAGGTGCTGCATGGTTGTCGTCAGCTCGTGT
1051 CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCGCGTGT
1101 TGCCAGCGGTTCGGCCGGGCACTCACGCTGAGACTGCCGGTGACAAACCG
1151 GAGGAAGGTGGGGATGACGTCAAATCCGCATGGCCTTGATGTCCTGGGCC
1201 ACACACGTGCTACAATGGCGCCGACAACGGGCCGCGACCCCGCGAGGGGC
1251 AGCGAATCCTTCAAACGGCGTCTCAGTTCGGATTGCAGGCTGCAACCCGC
1301 CTGCATGAAGCCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGA
1351 ATTCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGC
1401 CACACCCGAAGCCCGGTCGGTCGAACCCTTAGGGGGCGACCCCGTCGACG
1451 GTGGGGCGGATGATTGGGGTGAAGTCCTAGCAAGGTAGCCGTATCGGACG
1501 GTGCGGGTGGATC

Figure 4.1 16S rDNA partial nucleotide sequence of bacterial strain 4G; partial gene 1513 nucleotides (accession AY371272)

1 AACGGACCCTTTGGGGTCAGCGGCGGACGGGTAGTACACGTGGCAATCTG
51 CCGAGCAGACCCCGGAATAACGCCTGGAAACGGGTGCTAATGCCGGATAG
101 GCAGCGAGGAGGCATCTTCTTGGCCTGGGAAAGGTGCAACTTGCACCACT
151 GTTCGAGGAGCCCGCGGCGCATTAGCTTAGTTTGGTTAAGGTGAAGGCTT
201 ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCAACACCT
251 GGGACTGAGACACAGGCCCAGACTCCTACGGAAGGCAGCAGTAGGGAATC
301 TTCCGGCAATTGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGCGAAGA
351 AGGCCTTCGGGTTGTAAAAGCTTCAGTCACTCGGGAAGAGCGACCTAAGG
401 AGTGGAAAGCCTTAGGGGAGACGGTACCCGAGGGGAGGAAGCCCCGGCAA
451 ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAAGCGTTGTTCCG
501 GAATCACTGGGCGTAAAGCGTGCGTAGGCGGTTTTTTGGGTCTGGGGTGG
551 AAAGTTCCAGGGCTCAACCTTGAGAATGCCTTGGAAACTAGAAGACTTGA
601 GTGCTGGAGAAGGCAAGGGCGAATTCCACGTGTAGCGGTGAAATGCGTAG
651 AGATGTGGAGGAATACCAGTGGGCGAAGGCGCCCTTTGCTGGACAAGTGA
701 CTGACGCTGAGGCACGAAAGCGTGGGGAGCAAAACAGGATTAGAATACCC
751 TGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGGTCATAC
801 TCTCAGTGCCGAAGGAAACCCAATAAGCACTCCGCCTGGGGAGTACGGTC
851 GCAAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGA
901 GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGAC
951 ATCCCTCTGACCGGTACAGTAGATGGGACCTTCCCTTCGGGGCAGAGGAG
1001 ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTAGAGATGTTGGGTTA
1051 AGTCCCGCAACGGAGCGCAACCCTTGATCTGTGTTACCAGGCACGTGATG
1101 GGTGGGGACTCACAGGTGACTGCTCCGGCGTAAGTCGGAGGAAGGTGGGG
1151 ATGACGTCAAAATCATCATGCCCTTTATGTCCTGGGCCGACACACGTGCT
1201 ACAATGGGCGGTACAACGGGAAACCGAGACCGCGAGGTGGAGCGAAACCC
1251 TAAAAGCCGTTCGTAGTTCGGATTGCAGGCTGCAAACTCGCCTGCATGAA
1301 GCCGGAATTGCCTAGTAATCGCGGGATCAGCATGCCGCGGTGAATCCGTT
1351 TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGACAACACC
1401 CGAAGTCGGTTGAGGTAACCTTTTGGGGCCAGCCGCCGAAGGAGGTCGAG
1451 TGCCTGAAA

Figure 4.2 16S rDNA nucleotide sequence of bacterial strain 5C; partial gene 1459 nucleotides (AY371273)

1 GTTACGGGCCTTTTGGGGTCACGCGGACGGGTAGTACACGTGGCAATCTC
51 CGACAGACCCGGAATAACGCCTGGAAACGGGTGCTAATGCCGGATAGGCA
101 GCGAGGAGGCATCTTCTTGCCTGGGAAAGGTGCAACTTGCACCACTGTTC
151 GAGGAGCCCGCGGCGCATTAGCTAGTTGGTAAAGGTGAAGGCTTACCAAG
201 GCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACACTTGGGACTG
251 AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
301 TGGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGCCTTCG
351 GGTTGTAAAGCTCAAGTCACTCGGGAAGAGCGGACCTAAGGAGTGGAAAG
401 CCTTAGGGGAGACGGTACCGAGGGAAGGAAGCCCCGGCAAACTACGTGCC
451 AGCAGCCGCTGGTAATACGTAGGGGGCAAGCGTTGTTCCGGAATCCCTGG
501 GCGTAAAGCAGTGCGTAGGCGGTTTTTTGGGTCTGGGGTGAAAGTTCCAG
551 GGCTCAACCTTGAGAACTCCTTGGAAACTAGAAGACTTGAGTGCTGGAGA
601 GGCAAGGGCGAATTCCACGTGTAGCGGTGTAATGCGTAGAGATGTGGAGG
651 AATACCAGTGGCGAAGGCGACCTTGCTGGACAAGTGACTGACGCTGAGGC
701 ACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
751 TAAACGATGAGTGCTAGGTGTTGGGGGGTCATACTCTCAAGTGCCGAAGG
801 AAACCCAATAAGCACTCCGCCTGGGGAGTACGGGTCGCAAGACTGAAACT
851 CAAAGGAATTGACGGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAAT
901 TCGAAGCAACGCCGAAGAACCTTACCAGGGCTTGACATCCCTCTGACCGG
951 TACAGAGATGGACCTTCCCTTCGGGGCAGAGGAGACAGGTGGTGCATGGT
1001 TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
1051 AACCCTTGATCTGTGGTTACCAGCACGTGATGGTGGGGACTCACAGGTGA
1101 CTTGCCGGCGTAAGTCGGAGGAAGGTTGGGGATGACGTCAAATCATCATG
1151 CCCTTTATGTCCTGGGCGACACACGTGCTACAATGGGGCGGTACAACGGG
1201 AAGCGAGACCGCGAGGTGGAGCGAAACCTAAAAGCCGTTCGTAGTTCGGG
1251 ATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATTGCTAGTAATCGCG
1301 GATCAGCATTGCCGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCC
1351 GTCACACCATGGGAGTTGACCAACACCCGAAGTCGGTGAGGTAACCTTTT
1401 GGGGCCAGCCGCCGAAGGTGGGGTCGATGACCTGGGGTGAAGTCGTAACA
1451 AGGTAACCGTA

Figure 4.3 16S rDNA nucleotide sequence of bacterial strain Cligga; partial gene 1461 nucleotides (AY371274)

4.3 Similarity matrixes of 16S rDNA of isolated bacteria and other closely related bacteria

4.3.1 Introduction

Sequence comparisons (BLAST searches) were carried out with all available 16S rDNA nucleotide sequences available in NCMB Genbank Database, using the 4G, 5C and Cligga sequences illustrated in Figs 4.1, 4.2 and 4.3. The highest sequence identities were noted and are represented in Tables 4.1 and 4.2. Similarity matrixes were constructed using comparisons between the 16S rDNA data of all closely related bacteria and are shown in Tables 4.3 and 4.4. Similarity matrixes can usually be used to determine the main phylogenetic group to which an unidentified bacterium might belong (Stackebrandt & Rainey, 1995). Sequence similarities between genera have been found to range from 86% to 91%, while the same species within each genus usually share over 97% similarity (Benlloch *et al*, 1995).

4.3.2 Sequence similarities obtained for isolated strains 4G, 5C and Cligga

Sequence similarity of strain 4G with closely related bacteria and G+C content of 16S rDNA is shown in Table 4.1. This sequence analysis of the hypervariable region of the 16S rRNA gene of strain 4G shows highest identities of 96% with *Sulfobacillus sibiricus* (AY079150), 95% with *Sulfobacillus montserratensis* (AY007663) and 94% with *Sulfobacillus yellowstonensis* (AY007665). Analysis using 1200 nucleotides of the 16S rDNA produced 94% identity with *S. yellowstonensis* and other *Sulfobacillus* sp. which have not been properly identified. This result indicates that strain 4G might be a novel species of the genus *Sulfobacillus*.

Sequence similarity of strains 5C and Cligga with closely related bacteria and G+C content of 16S rDNA is shown in Table 4.2. The hypervariable region of the 16S rDNA of strain 5C showed highest identity with *Alicyclobacillus* sp. strain AGC-2 (AF450135) and an unidentified Bacterium GSM (AY007662) with a sequence similarity of 95%. Analysis with 1200 nucleotides of 16S rDNA gave identities of 95% and 94% with these bacteria, respectively. Since these identities are under the similarity

expected within species (97%) this result indicated that strain 5C might be a new species from the genus *Alicyclobacillus*.

Analysis of the hypervariable region of 16S rDNA from strain Cligga produced identities of 97% with *Alicyclobacillus* sp. strain AGC-2 (AF450135) and 96% with unidentified Bacterium GSM. Both of these bacteria showed 97% identity with Cligga when 1200 nucleotides of 16S rDNA was used as the query sequence. This indicates that strain Cligga may also be a strain or sub-species of the type represented by *Alicyclobacillus* strain AGC-2 and Bacterium GSM.

Table 4.1 Showing the G + C content (mol%) of the 16S rDNA of bacteria that are closely related to isolated Bacterium 4G (based on 16S sequences from Genbank and Ribosomal Database Project software). Sequence similarity is based on the hypervariable region and of the first 1200 nucleotides of Bacterium 4G 16S rDNA to closely related strains (based on BLAST search)

Bacteria and (accession number)	**G+C content** of 16S rDNA (mol%)	Sequence similarity to **hypervariable** region of 16S rDNA of **4G** (%)	Sequence similarity to **1200bp**16S rDNA of **4G** (%)
Bacterium 4G	63.25	100	100
Sulfobacillus yellowstonensis (AY007665)	64.89	94	94
Sulfobacillus sp. strain NC (AY12160)	65.09	92	94
Sulfobacillus sp. strain YTF3 (AF507964)	65.16	92	94
Sulfobacillus sp. strain GG6/1 (AY140234)	65.04	92	94
Sulfobacillus sp. strain Fras 1 (AF213055)	62.17	88	91
Sulfobacillus sp. strain Y0017 (AY140239)	62.39	93	93
Sulfobacillus montserratensis (AY007663)	61.59	95	93
Sulfobacillus sp. strain K55 (AF460984)	63.55	94	93
Bacterial sp. OS77 (X86775)	62.6	93	93
Sulfobacillus ambivalens (AY007664)	61.43	92	92
Sulfobacillus sp. strain G2 (AY140233)	62.93	93	93
Sulfobacillus thermosulfidooxidans (X91080)	62.93	94	93
Sulfobacillus sibiricus (AY079150)	62.52	96	93
Sulfobacillus thermosulfidooxidans(U75648)	62.15	93	91
Sulfobacillus acidophilus (AF050169)	63.6	88	92

Table 4.2 Showing the G + C content (mol%) of the 16S rDNA of bacteria that are closely related to isolated Bacteria 5C and Cligga (based on 16S sequences from Genbank and The Ribosomal Database Project software). Sequence similarity is based on the hypervariable region and of the first 1200 nucleotides of Bacteria 5C and Cligga 16S rDNA to closely related strains (based on BLAST search). ND – not determined.

Bacterium name and (accession number)	**G+C content** of16S rDNA (mol%)	Sequence similarity to **hypervariable** region of 16S rDNA of **5C** (%)	Sequence similarity to **1200bp**16S rDNA of **5C** (%)	Sequence similarity to **hypervariable** region of 16S rDNA of **Cligga** (%)	Sequence similarity to **1200bp** 16S rDNA of **Cligga** (%)
Bacterium 5C	57.23	100	100	95	95
Bacterium Cligga	57.36	95	95	100	100
Alicyclobacillus sp. strain AGC-2 (AF450135)	57.93	95	95	97	97
Bacterium GSM (AY007662)	57.98	95	94	96	97
Alicyclobacillus acioterrestris DSM 2498 (AB059675)	57.13	92	94	95	93
Alicyclobacillus acidoterrestris DSM 3922 (AJ133631)	57.35	92	94	96	93
Alicyclobacillus hesperidensis FR-11 (AJ133633)	57.71	88	93	88	92
Alicyclobacillus cycloheptanicus (AB042059)	59.33	88	93	89	92
Alicyclobacillus acidocaldarius DSM 449 (AB059671)	59.83	90	93	88	93
Gram +ve heterotrophic acidophile Y004 (AY140236)	58.36	92	92	95	92
Sulfobacillus disulfidooxidans (U34974)	59.22	94	91	96	92
Alicyclobacillus herbarius (AB042055)	60.04	93	90	92	92
Alicyclobacillus hesperidum DSM 12766 (AB059679)	57.48	88	93	88	92
Alicyclobacillus sp. TA 67T (AB059677)	56.93	90	89	90	91
Sulfobacillus sp. YTH1 (AF031645)	58.16	90	91	90	91

Table 4.3 Similarity matrix for isolated bacterium 4G and some closely related bacteria. Matrix based on BLAST searches of EMBL GENBANK with partial 16S rDNA sequences (1200 nucleotides).

	Sequence identities (%) obtained from BLAST searches															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1 Strain 4G	100	94	94	94	94	91	93	93	93	93	92	93	93	93	91	92
2 *Sulfobacillus yellowstonensis* (AY007665)	94	100	97	97	99	91	92	94	92	92	92	93	92	92	94	95
3 *Sulfobacillus* sp. strain NC (AY12160)	94	97	100	98	99	91	93	94	92	92	92	92	92	92	93	96
4 *Sulfobacillus* sp. strain YTF3 (AF507964)	94	97	98	100	99	91	93	94	92	92	92	92	92	92	93	95
5 *Sulfobacillus* sp. strain GG6/1 (AY140234)	94	99	99	99	100	92	93	94	92	92	92	93	92	92	93	95
6 *Sulfobacillus* sp. strain Fras 1 (AF213055)	91	91	91	91	91	100	91	90	94	94	90	91	91	91	91	90
7 *Sulfobacillus* sp. strain Y0017 (AY140239)	93	92	93	93	93	91	100	97	95	94	97	96	96	96	95	90
8 *Sulfobacillus montserratensis* (AY007663)	93	94	94	94	94	91	97	100	94	93	97	95	95	95	94	91
9 *Sulfobacillus* sp. strain K55 (AF460984)	93	92	92	92	92	94	95	94	100	98	94	97	96	96	96	89
10 Bacterial sp. OS77 (X86775)	93	92	92	92	92	94	95	93	98	100	93	96	95	95	95	89
11 *Sulfobacillus ambivalens* (AY007664)	93	92	92	92	92	90	97	97	94	93	100	94	94	95	93	90
12 *Sulfobacillus* sp. strain G2 (AY140233)	93	93	92	92	92	91	96	95	97	96	94	100	99	98	98	89
13 *Sulfobacillus thermosulfidooxidans* (X91080)	93	93	92	92	92	91	96	95	96	95	94	99	100	98	97	89
14 *Sulfobacillus sibiricus* (AY079150)	93	92	92	92	92	91	96	95	96	95	95	98	98	100	97	89
15 *Sulfobacillus thermosulfidooxidans*(U75648)	91	94	93	93	93	91	95	94	96	95	93	98	97	97	100	92
16 *Sulfobacillus acidophilus* (AF050169)	92	95	96	95	95	90	90	91	89	89	90	89	89	89	92	100

Table 4.4 Similarity matrix for isolated bacteria 5C and Cligga and some closely related bacteria. Matrix based on BLAST searches of EMBL GENBANK with partial 16S rDNA sequences (1200 nucleotides). ND – not determined

	Sequence identities (%) obtained from BLAST searches														
Bacterium name and (accession number)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1 Strain 5C	100	95	95	94	94	94	93	93	93	92	91	90	93	89	91
2 Strain Cligga	95	100	97	97	93	93	92	92	93	92	92	92	92	91	91
3 *Alicyclobacillus* sp. strain AGC-2 (AF450135)	95	97	100	99	95	93	94	93	92	95	95	93	93	94	93
4 Bacterium GSM (AY007662)	94	97	99	100	94	92	93	93	91	89	94	93	93	93	92
5 *Alicyclobacillus acioterrestris* DSM 2498 (AB059675)	94	93	95	94	100	98	97	95	96	93	93	ND	96	95	93
6 *Alicyclobacillus acidoterrestris* DSM 3922 (AJ133631)	94	93	93	92	99	100	97	93	96	94	93	ND	97	96	93
7 *Alicyclobacillus hesperidensis* FR-11 (AJ133633)	93	92	94	93	97	97	100	93	95	93	93	ND	99	96	ND
8 *Alicyclobacillus cycloheptanicus* (AB042059)	93	92	93	93	95	93	93	100	93	93	94	92	93	94	92
9 *Alicyclobacillus acidocaldarius* DSM 449 (AB059671)	93	93	92	91	96	96	95	93	100	93	96	95	95	95	92
10 Gram +ve heterotrophic acidophile Y004 (AY140236)	92	92	95	89	93	94	93	93	93	100	93	93	93	93	99
11 *Sulfobacillus disulfidooxidans* (U34974)	91	92	95	94	93	93	93	94	96	93	100	93	93	92	93
12 *Alicyclobacillus herbarius* (AB042055)	90	92	93	93	ND	ND	ND	92	95	95	93	100	ND	95	92
13 *Alicyclobacillus hesperidium* DSM 12766 (AB059679)	93	92	93	93	96	97	99	93	95	93	93	ND	100	96	92
14 *Alicyclobacillus* sp. TA 67T (AB059677)	89	91	94	93	95	96	96	94	95	93	92	95	96	100	92
15 *Sulfobacillus* sp. YTH1 (AF031645)	91	91	92	93	93	93	ND	92	92	99	93	92	92	92	100

4.4 Phylogenetic tree construction for the isolated acidophilic bacteria and other related bacteria

4.4.1 Introduction

The phylogenetic trees constructed during this study are the visualisation of the phylogenetic relationships of bacteria expressed in dissimilarity values. The terminal nodes represent the 16S molecules of the analysed bacteria while the internal nodes represents a common stage in the evolution of these molecules. In unrooted trees only the interrelationships of the included bacteria are indicated, whereas in rooted trees the position of the common ancestor is indicated and the calculated order at which the bacteria evolved is displayed (Stackebrandt & Rainey, 1995). Evolutionary distances are indicated by the sum of horizontal branch lengths and bacteria tend to cluster at bootstrap confidence levels of 97% - 100%, i.e. bacteria of the same species.

4.4.2 Phylogenetic trees showing the relationships of the isolated acidophilic bacteria with other similar bacteria

In the case of bacterial strain 4G both the 600 nucleotide and 1200 nucleotide phylograms (Figures 4.4 and 4.5 respectively) show the distinct clustering within the Genus *Sulfobacillus*. One cluster groups *S. montserratensis*, *Sulfobacillus* sp. Y0017 and *S. ambivalens* together, and these bacteria were all isolated from geothermal areas on the Island of Montserrat (see Table 4.5 for isolation locales), further indication of a close line of descent. *Sulfobacillus* sp. Fras1 and strain 4G are the most distantly related to the other members of the Genus *Sulfobacillus* but are also distantly related to each other based on branch lengths and consolidated by the similarity matrix in Table 4.3.

For the bacterial strains 5C and Cligga both the 600 nucleotide and 1200 nucleotide phylograms (Fig. 4.6 and Fig 4.7 respectively) are quite similar and show distinct clustering of species within the genus *Alicyclobacillus*. They show that these two isolates are most closely related to *Alicyclobacillus* sp. AGC-2 and bacterium GSM (as shown by the high similarity matrix values, Table 4.4). *Alicyclobacillus* sp. AGC-2 was

isolated from a thermal spring in Alaska in similarity to many of the represented species that have been isolated from geothermal areas (see Table 4.6).

The relatedness of the three isolated bacterial strains to both the genera *Sulfobacillus* and *Alicyclobacillus* was compared by construction of rooted phylogenetic trees (Figures 4.8 and 4.9) using *At. ferrooxidans* as an out-group to root the tree.

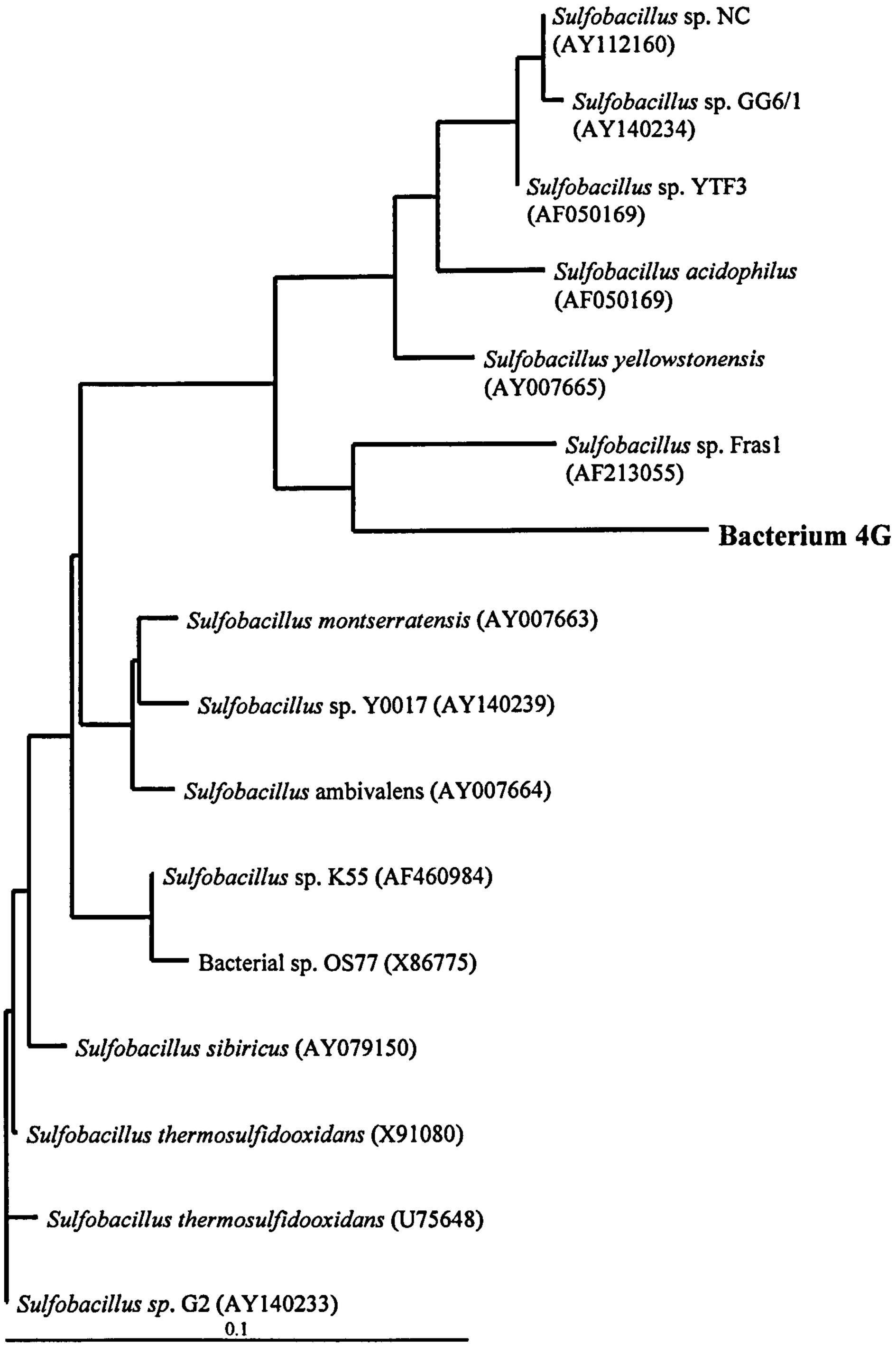


Figure 4.4 Unrooted evolutionary distance phylogram that illustrates relationships between the isolated bacterium 4G and other closely related bacteria based on the hypervariable region of 16S rDNA. Tree constructed using comparison of 600 nucleotides aligned using Clustal W and TreeView tree drawing software. The bar indicates substitutions per nucleotide. Accession numbers are shown in brackets.

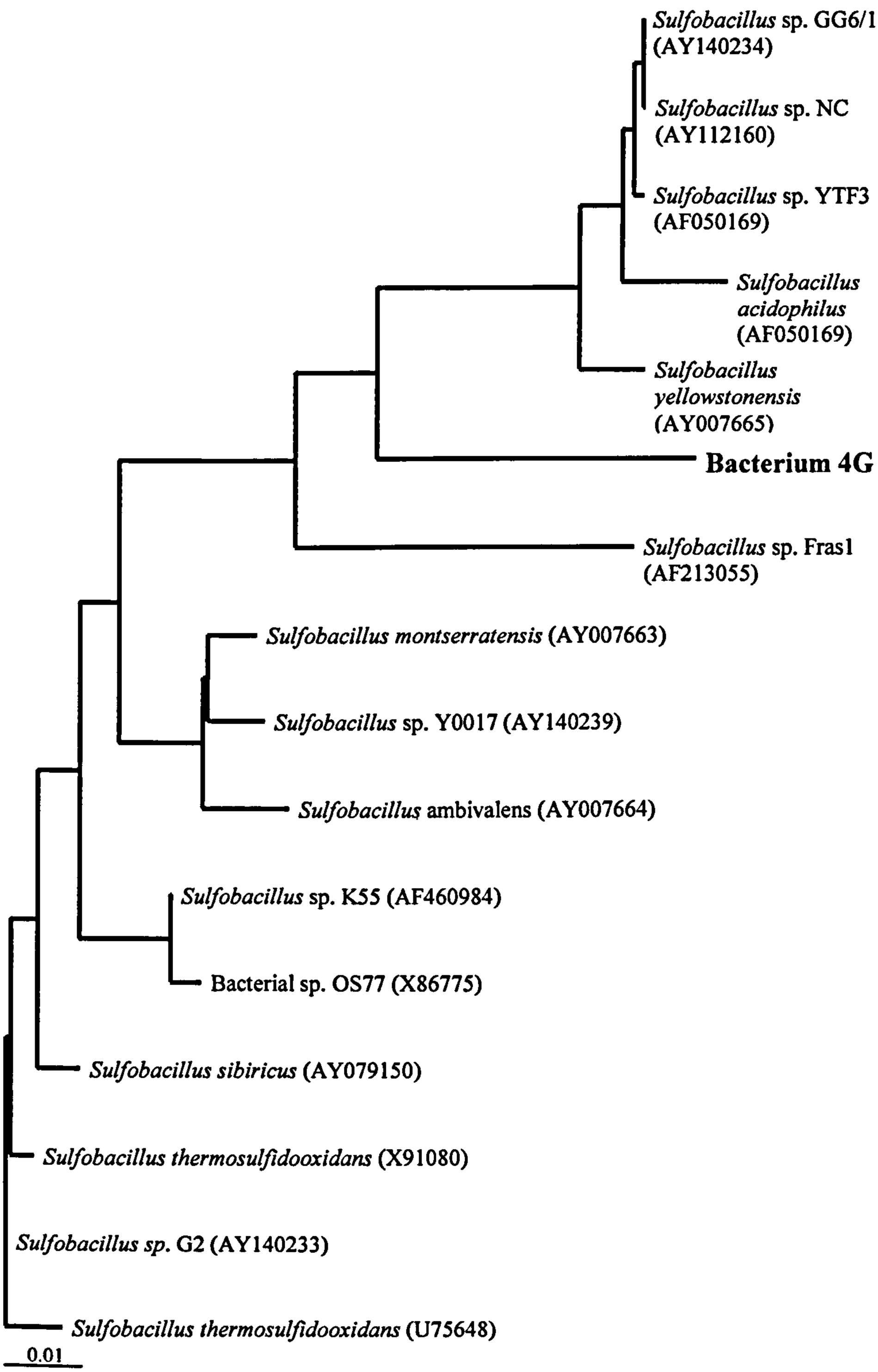


Figure 4.5 Unrooted evolutionary distance phylogram illustrating relationships between the isolated bacterium 4G and other closely related bacteria. Tree constructed using comparison of 1200 16S rDNA nucleotides aligned using Clustal W and TreeView tree drawing software. The bar indicates substitutions per nucleotide. Accession numbers are shown in brackets.

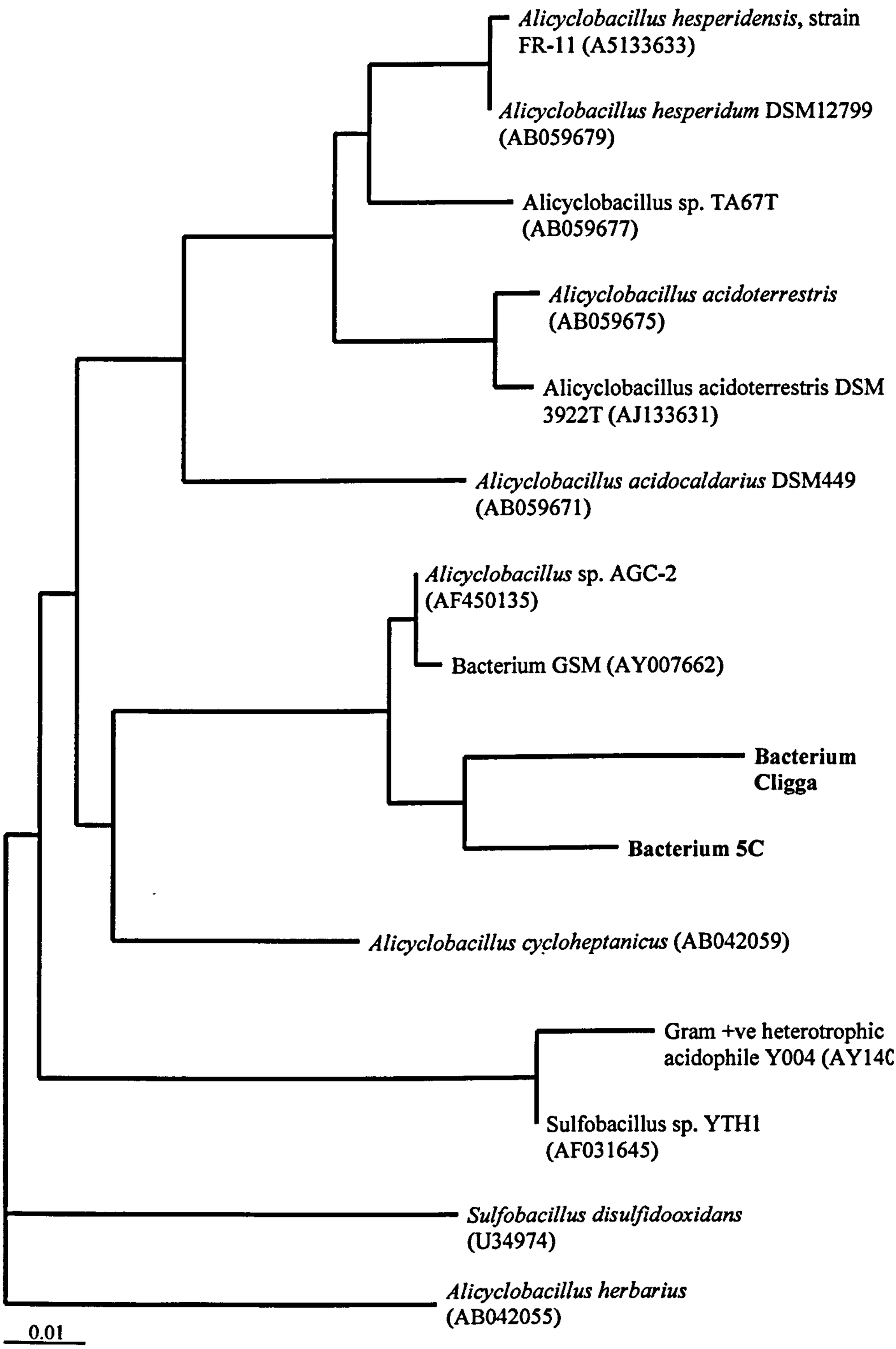


Figure 4.6 Unrooted evolutionary distance phylogram that illustrates relationships between the isolated bacteria 5C and Cligga and other closely related bacteria based on the hypervariable region of 16S rDNA. Tree constructed using comparison of 600 nucleotides aligned using Clustal W and TreeView tree drawing software. The bar indicates substitutions per nucleotide. Accession numbers are shown in brackets.

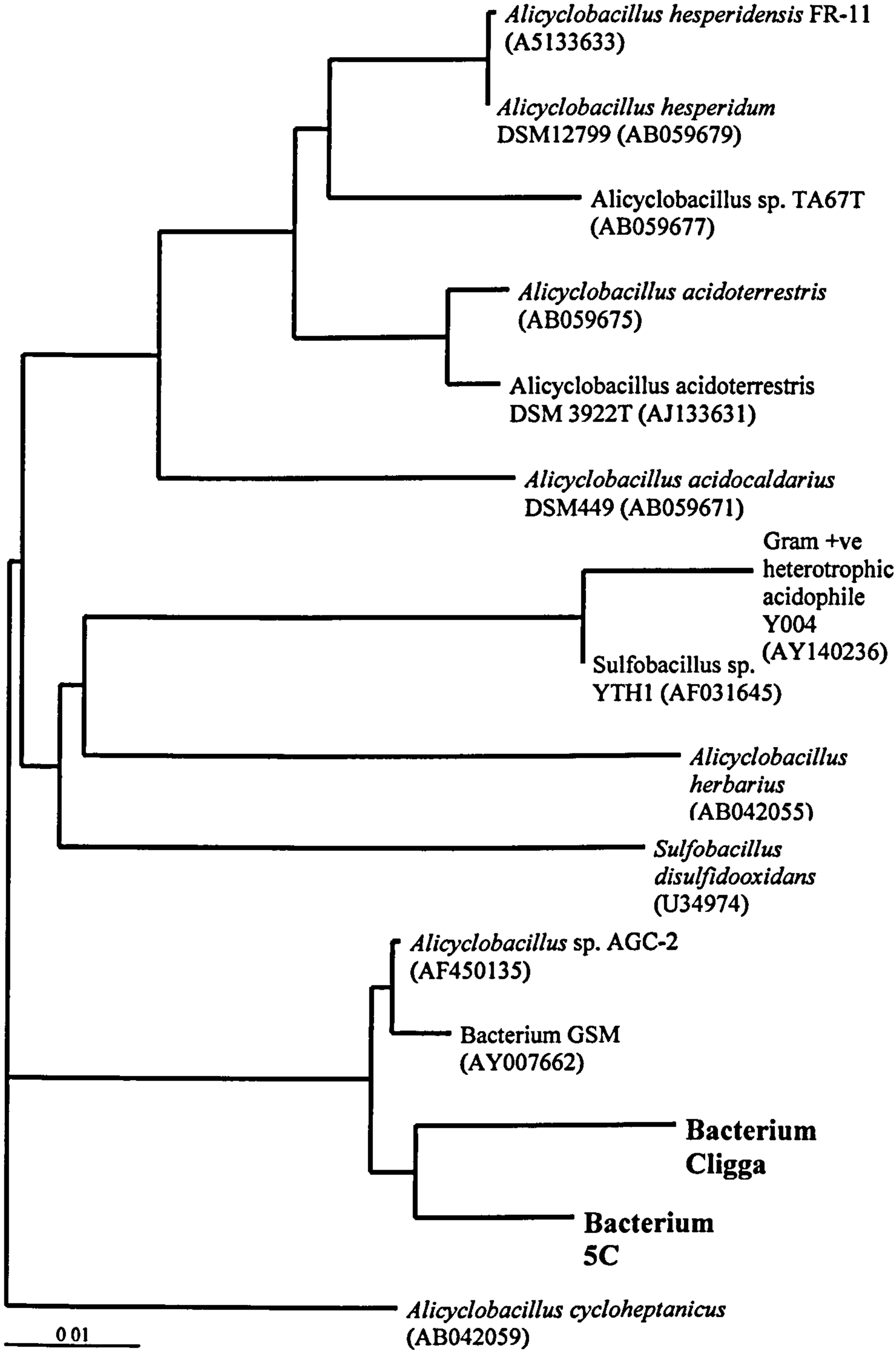


Figure 4.7 Unrooted evolutionary distance phylogram illustrating relationships between the isolated bacteria 5C and Cligga and other closely related bacteria. Tree constructed using comparison of **1200** 16S rDNA nucleotides aligned using Clustal W and TreeView tree drawing software. The bar indicates substitutions per nucleotide. Accession numbers are shown in brackets.

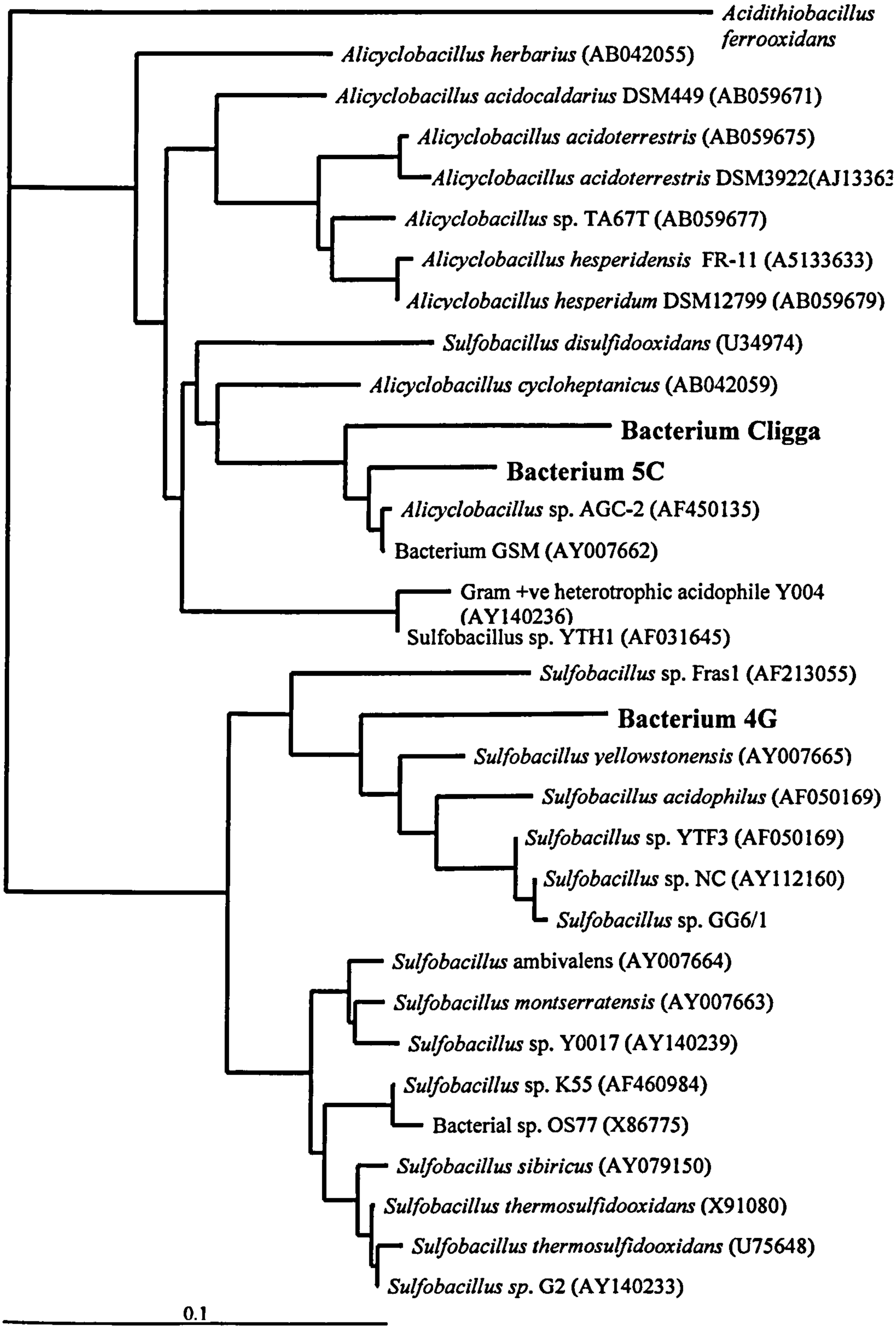


Figure 4.8 Rooted evolutionary distance phylogram which illustrates relationships between the isolated bacteria 4G, 5C and Cligga and other closely related bacteria based on the hypervariable region of 16S rDNA. Tree constructed using comparison of 600 nucleotides aligned using Clustal W and TreeView tree drawing software. The bar indicates substitutions per nucleotide. Accession numbers are shown in brackets. The tree was rooted using *Acidithiobacillus ferrooxidans* as the out-group.

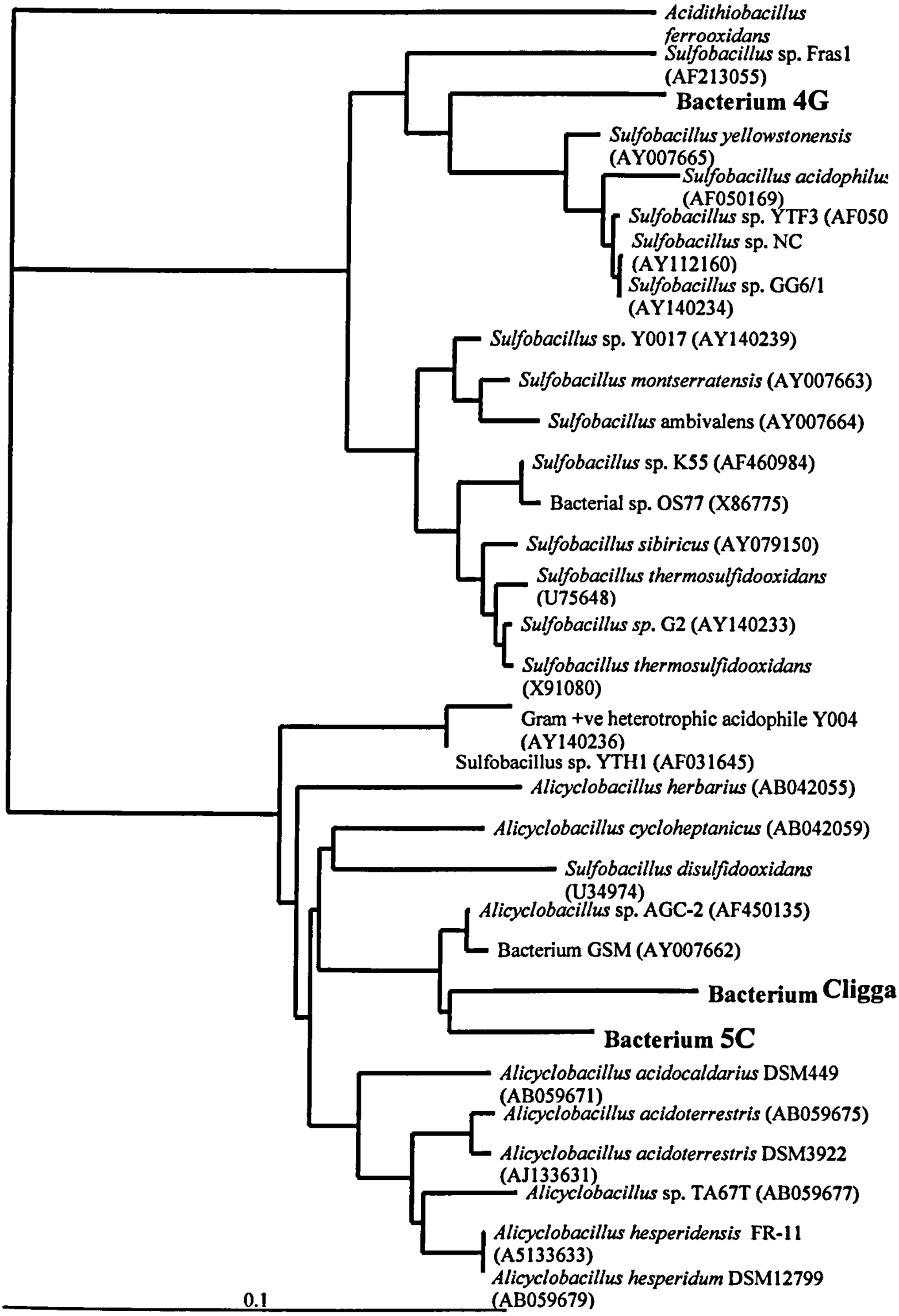


Figure 4.9 Rooted evolutionary distance phylogram that illustrates relationships between the isolated bacteria 4G, 5C and Cligga and other closely related bacteria. Tree constructed using comparison of **1200** 16S rDNA nucleotides aligned using Clustal W and TreeView tree drawing software. The bar indicates substitutions per nucleotide. Accession numbers are shown in brackets. The tree was rooted using *Acidithiobacillus ferrooxidans* as the out-group.

Table 4.5 Isolation environments and references of bacterial species closely related to isolated bacterium 4G

Bacteria and accession number of 16S rDNA sequence	Type of isolation environment	Reference
Strain 4G (AY371272)	Tidal estuary with pyritic tailings contamination	This study
Sulfobacillus yellowstonensis (AY007665)	Geothermal area of Yellowstone park	Unpublished
Sulfobacillus sp. strain NC (AY12160)	Pilot plant stirred-tank bioleaching operation	Okibe *et al*, 2003
Sulfobacillus sp. strain YTF3 (AF507964)	Not reported	Unpublished
Sulfobacillus sp. strain GG6/1 (AY140234)	Geothermal area of Montserrat	Unpublished
Sulfobacillus sp. strain Fras 1 (AF213055)	Acidic Cave-Wall Biofilms Located in the Frasassi Gorge, Italy	Vlasceanu *et al*, 2000
Sulfobacillus sp. strain Y0017 (AY140239)	Geothermal area of Montserrat	Unpublished
Sulfobacillus montserratensis (AY007663)	Geothermal area of Montserrat	Unpublished
Sulfobacillus sp. strain K55 (AF460984)	Not reported	Norris *et al*, 1996
Bacterial sp. OS77 (X86775)	natural acidic environment	Battaglia-Brunet *et al*, 2002
Sulfobacillus ambivalens (AY007664)	Geothermal area of Montserrat	Unpublished
Sulfobacillus sp. strain G2 (AY140233)	Geothermal area of Montserrat	Unpublished
Sulfobacillus thermosulfidooxidans (X91080)	Not reported	Golovacheva & Karavaiko, 1978
Sulfobacillus sibiricus (AY079150)	Not reported	Unpublished
Sulfobacillus thermosulfidooxidans(U75648)	Not reported	Norris *et al*, 1996
Sulfobacillus acidophilus (AF050169)	Not reported	Norris *et al*, 1996

Table 4.6 Isolation environments and references of bacterial species closely related to isolated bacteria 5C and Cligga

Bacterium name and accession number of 16S rDNA sequence	Type of isolation environment	Reference
Strain 5C (AY371273)	Tidal estuary with contaminated pyritic mine tailings	This study
Strain Cligga (AY371274)	Tidal caves, disused tin mine	This study
Alicyclobacillus sp. strain AGC-2 (AF450135)	Thermal spring, Alaska	Unpublished
Bacterium GSM (AY007662)	Not reported	Unpublished
Alicyclobacillus acidoterrestris DSM 2498 (AB059675)	Not reported	Unpublished
Alicyclobacillus acidoterrestris DSM 3922 (AJ133631)	solfataric soils of Sao Miguel in the Azores	Albuquerque *et al*, 2000
Alicyclobacillus hesperidensis FR-11 (AJ133633)	solfataric soils of Sao Miguel in the Azores	Albuquerque *et al*, 2000
Alicyclobacillus cycloheptanicus (AB042059)	herbal tea	Goto *et al*, 2002
Alicyclobacillus acidocaldarius DSM 449 (AB059671)	Not reported	Unpublished
Gram +ve heterotrophic acidophile Y004 (AY140236)	Geothermal acidic site in Yellowstone National park	Johnson *et al*, 1997
Sulfobacillus disulfidooxidans (U34974)	Wastewater sludge	Dufresne *et al*, 1996
Alicyclobacillus herbarius (AB042055)	Herbal tea made from the flowers of hibiscus	Goto *et al*, 2002
Alicyclobacillus hesperidum DSM 12766 (AB059679)	Not reported	Unpublished
Alicyclobacillus sp. TA 67T (AB059677)	Not reported	Unpublished
Sulfobacillus sp. YTH1 (AF031645)	Geothermal acidic site in Yellowstone National park	Johnson *et al*, 1997

4.5 Discussion

Within the genera represented by the data shown there are many species of closely related bacteria, each with distinct phylogenetic and physiological features. However, they can be seen to cluster into clearly defined groups within genera, which are often characterised by similar isolation environments, highlighting evolutionary processes taking place in distinct environs.

In this study, the partial 16S rDNA sequences of three, halotolerant, iron-oxidising, bacterial strains were determined and analysed for identification and to elucidate the relationship with other similar bacteria. It was found that the 16S rDNA of strain 4G showed a low sequence similarity with those in the databases. The highest identity was observed with *S. sibiricus* (96%) for which there is no physiological data available in the literature and *S. montserratensis* (95%), which was isolated from a geothermal site on the Island of Montserrat. Despite the high temperature of the isolation locale (50-58°C) *S. montserratensis* is a mesophilic gram positive spore forming bacteria and there is currently only one other mesophilic member of the Genus *Sulfobacullus* reported in the literature, *S. ambivalens*. Since strain 4G is mesophilic T_{opt} 37°C it is not unexpected that among this genus it would be most closely related to another mesophilic bacterium.

Strains 5C and Cligga were found to belong to the Genus *Alicyclobacillus* having 95% and 97% identity respectively with the 16S rDNA of Alicyclobacillus sp. strain AGC-2 which was isolated from a thermal spring in Alaska. The strains 5C and Cligga also showed 95% and 96% identity respectively with the unidentified bacterium GSM. There have been no reports to date of the characteristics of these bacterial species (*Alicyclobacillus* sp. strain AGC-2 and bacterium GSM) and therefore adequate phenotypic comparisons cannot be made with 5C and Cligga. However, most of the related *Alicyclobacillus* species differ in their metabolic characteristics as they are mostly obligate heterotrophs, in contrast, 5C and Cligga have been found to grow autotrophically, heterotrophically and mixotrophically. Therefore they may represent a previously unknown sub-group within the Genus *Alicyclobacillus*.

The rooted phylograms of all closely related species to 4G, 5C and Cligga showed the common descent of the genera *Sulfobacillus* and *Alicyclobacillus* and this has been reported based on phenotypic and genetic analysis by Tourova *et al* (1994). Tourova *et al* noted this linkage shown by sequence signatures at certain positions for these two genera based on alignments of 44 bacterial species.

The 16S rDNA for the isolated strain 4G data presented in this chapter along with physiological and morphological data reported in previous chapters suggests bacterium 4G represents a new species within the Genus *Sulfobacillus*. The name *Sulfobacillus halodurans* sp. nov. is tentatively proposed for this species. Using the reported data it is also suggested that strain 5C represents a new species within the Genus *Alicyclobacillus*. The name *Alicyclobacillus halodurans* sp. nov. is tentatively proposed for this species. Strain Cligga also represents a species within the Genus Alicyclobacillus within the same species represented by Alicyclobacillus sp. strain AGC-2 (AF450135) and bacterium GSM (AY007662). Further characteristics of these two related strains must be elucidated before a species name can be proposed for strain Cligga.

However, other taxonomic techniques including DNA:DNA homology, whole-cell protein comparisons, analysis of fatty acid composition and biochemical tests (such as the detection of ω-alicyclic fatty acids for identification of members of the Genus Alicyclobacillus) should be carried out in order to unequivocally assign the phylogenetics and specific names for these three bacterial strains.

Chapter Five

Bioleaching potential of the isolated halotolerant bacteria at high salinity

Chapter Five: Results

5.1 Introduction

Methods of extraction of metals from metaliferrous ores have traditionally involved chemical and physical processes (including pressure leaching and cyanide extraction). However, mining companies are increasingly looking for more environmentally friendly and economic methods of metal extraction. This is due, in part, to the increasing amount of legislation regarding mine waste disposal and environmental protection and the increasing capital costs of traditional extraction methods. In addition, many low-grade ores are difficult to treat with existing chemical methods. Therefore, mining companies are constantly on the look-out for new processes that would decrease their capital costs and allow them to comply with necessary legislation relatively inexpensively. Bioleaching is a technology that has increasingly been utilised by some mining companies as a complementary process or as an alternative to traditional extraction methods.

Bioleaching is the use of iron or sulphur-oxidising, acidophilic bacteria to solubilise metals from mineral ores. This process harnesses the natural metabolic abilities of these bacteria, which use iron and sulphur compounds as an electron source for energy production and produce sulphuric acid as a product of metabolism.

Biooxidation is a complex process involving both biological and abiotic factors, which are inextricably linked. Many researchers have presented evidence for two modes of action during such leaching processes (theory first proposed by Silvermann & Ehrlich, 1964) . The first mode is termed the 'direct' mechanism, in which the bacteria are direct contact with the ore particles, oxidising ferrous iron and reduced sulphur as part of energy acquisition and thereby enhancing the overall rate of dissolution. The 'in-direct' mechanism occurs when unattached planktonic cells produce ferric iron ions by the oxidation of ferrous iron in solution which then attack the ore particles and catalyse chemical leaching.

The interactions of the above mentioned mechanisms mean there is constant flux between the ferrous and ferric iron as the dominant oxidation state in the leachate. Research has shown that the ferric leaching and iron-oxidation kinetics of sulphide

minerals is dependent on the redox potential (ferric/ferrous iron ratio) of the bioleaching solution (Breed & Hansford, 1999, May et al, 1997 and Ruitenberg et al, 1999). Favourable bioleaching conditions include a high redox potential and Eh measurements can therefore be used as an indication of the bioleaching conditions within a system and have been used as a direct measurement of the growth of iron-oxidising acidophilic bacteria (Fowler *et al*, 1999 and Pesic, 1993).

Many copper and gold mine sites around the world occur on island or coastal sites, for example the Lihir gold mine on the Island of Papua New Guinea. Also, many mines are contaminated with wind blown chloride and nitrate salts from nearby salt petre deposits and mining companies may want to use the resulting high salinity water or bore hole water as a lixivant resource. Therefore, the use of seawater as a lixivant in bioleaching processes would be of high economic importance and may give a significant process advantage for bioleaching operations in coastal areas.

However, traditional bioleaching microorganisms generally find even low levels of chloride salts toxic, and the contained salts in seawater generally inhibit growth and biooxidation processes. Therefore, the halotolerant, acidophilic iron/sulphur oxidising bacteria isolated in this study would give an economic advantage to a mine site such as Lihir, providing bioleaching rates were sufficient to give a significant process advantage.

The rate of a leaching process may potentially be enhanced by contained free chloride ions, which are corrosive, especially when in contact with pyritic ore (Personal communication David Barr, Rio Tinto Melbourne, 2000). The use of halotolerant bacteria would also allow the recycling of the biooxidation leachate and would therefore reduce the amount of potentially toxic waste being disposed of, thereby potentially saving money that is usually spent on complying with the legislation involved in mine waste disposal. In addition, this re-use of the leachate would decrease the environmental impacts of disposal of the mine waste. Heap bioleaching could also be carried out while the actual mine is being closed down and the land reclaimed, and can usually be incorporated into the surrounding countryside with the minimum of invasiveness.

All bioleaching experiments were performed using isolated bacteria cultured in 250 ml Erylenmeyer flasks with 100 ml of pyrite saline medium (PSM), with the addition of 2 % (w/v) ore sample instead of the pyrite and 30 g l^{-1} sea salts (see Materials and Methods Section 2.2.3). The initial pH of the medium was pH 2.0 and the flasks were incubated at a temperature of 37°C. Bioleaching experiments performed using *At. ferrooxidans* were carried out in PM medium containing 2 % (w/v) ore sample and at an initial pH of 2.0. No sea salts were added to this medium and these cultures were incubated at 28 °C. All media were sterilised by autoclaving at 121 °C for 15 minutes. The low pH of the medium ensured that a minimal amount of the ore material was oxidised during autoclaving. All experiments were carried out on an orbital shaker with a shaking speed of 180 rpm.

Experiments were performed in duplicate and averages were plotted with the error bars representing ± standard deviation from the mean values. All ferrous and iron dissolution values were corrected for oxidation of ferrous iron and dissolution of iron from the mineral ore that was not caused by growth of the test bacteria. This was done by subtraction of the increase or decrease in total or ferrous iron concentrations that had been calculated from means of duplicate uninoculated negative control flasks.

5.2 Bioleaching of Las Cruces Spanish copper ore by the isolated bacteria

5.2.1 Introduction

Las Cruces ore is a copper ore with a copper concentration of 3.84 %. It is mostly composed of chalcopyrite ($CuFeS_2$) along with other component minerals. It also contains relatively high concentrations of lead (9.854 mg g^{-1}), zinc (9.611 mg g^{-1}) and arsenic (4.506 mg g^{-1}) (Rio Tinto Technology Ltd; Technical Report).

5.2.2 Growth and iron dissolution kinetics of isolates grown on Las Cruces ore

All of the bacterial strains tested grew well on Las Cruces copper ore, at 2 % (w/v) ore pulp density and with a sea salt concentration of 30 gl^{-1} (3 % w/v) (Figure 5.1). All three strains exhibited short lag periods and reached stationary phase between 5 (5C) and 9 (4G) days. The exponential growth rate constants of the bacteria were as follows; 4G 0.86 day^{-1}, 5C 1.35 day^{-1}, and Cligga 1.03 day^{-1}. Generation times were; 4G 27.84 hours, 5C 17.76 hours, and Cligga 23.28 hours during log phase growth.

Ferrous iron concentration (Figure 5.2) remained relatively unchanged until day four when there was a decrease, probably because of the oxidation of the ferrous iron for energy acquisition during growth of the bacteria. This decrease continued for 4G until day seven when ferrous iron concentration increased sharply to a maximum of over 14 mM at day thirty, coinciding with the high rate of iron dissolution over the same period. The ferrous iron concentration in cultures of 5C and Cligga increased only slightly for the rest of the experiment.

Total iron in solution during bioleaching (Figure 5.3) remained steady from the beginning of the experiment until around day six when dissolution by 4G increased rapidly until the end of the experiment when there was a total of 1.91 gl^{-1} in solution. Dissolution of iron by 5C and Cligga increased only slightly to a maximum of 0.45 gl^{-1} and 0.44 gl^{-1} respectively. Iron dissolution by *At. ferrooxidans* was also examined for comparison purposes, though no sea salts were added to the growth medium. Iron dissolution by *At. ferrooxidans* also commenced around day six and increased to a maximum of 3.42 gl^{-1} at day thirty of the experiment. Iron dissolution as a percentage

of the total iron in the medium is shown in Figure. 5.4, with 4G extracting 22.69 % of the total iron as compared to 40.23 % extracted by the benchmark microorganism, *At. ferrooxidans*. However, it should be noted that under the high salinity growth conditions of 4G, 5C and Cligga strains, *At. ferrooxidans* would not grow. Average and exponential rates of iron dissolution are shown in Table 5.1, and again 4G exhibits the fastest rate of iron dissolution of the isolated bacteria.

Figure 5.5 shows the pH change in the cultures grown on the Las Cruces copper ore. The largest drop between initial and final pH was detected in cultures of *At. ferrooxidans*, indicating higher production of H_2SO_4 and this is associated with better growth in these cultures and better iron-oxidation which is demonstrated by Figure 5.3. Of the salt-tolerant bacteria, 4G exhibited the biggest drop in pH and also the highest rate of iron dissolution (Table 5.1). The change in redox potential during growth on Las Cruces ore is presented in Figure 5.6. All of the test cultures exhibited an increase in redox potential. The cultures of *At. ferrooxidans* showed the greatest increase in redox potential and this was expected due to the high rate of iron dissolution observed (redox potential is a measure of the ferric/ferrous iron ratio). Of the three salt-tolerant bacteria cultures of 4G exhibited the largest increase in redox potential and this was because the rate of growth and rate of iron dissolution was highest for this strain.

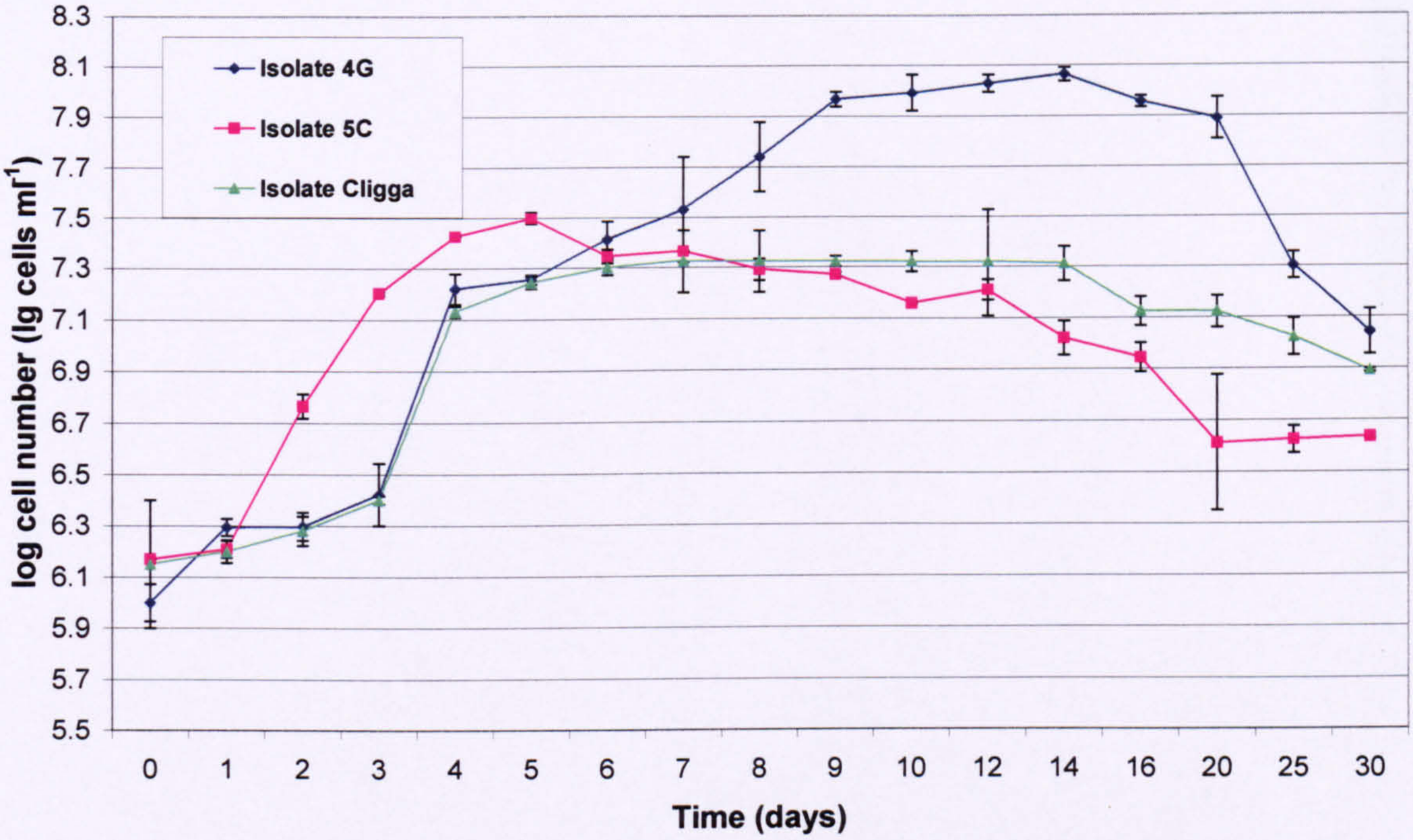


Figure 5.1 Growth of isolated bacteria on 2 % (w/v) Las Cruces chalcopyrite copper ore in medium with 30 gl^{-1} sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

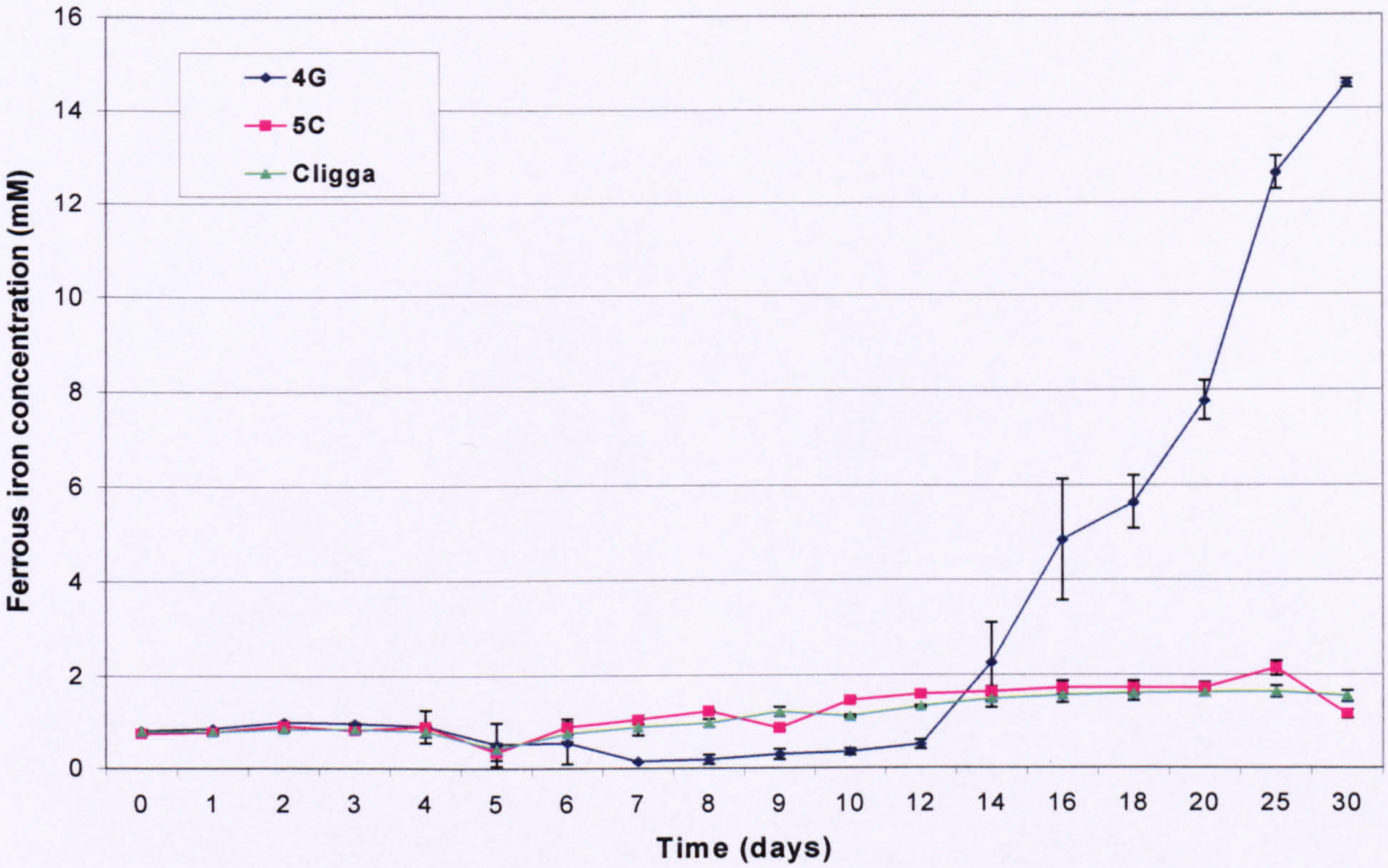


Figure 5.2 Change in ferrous iron concentration in cultures of isolated bacteria grown on Las Cruces chalcopyrite copper ore. Each datum point represents the mean ± standard deviation of duplicate cultures.

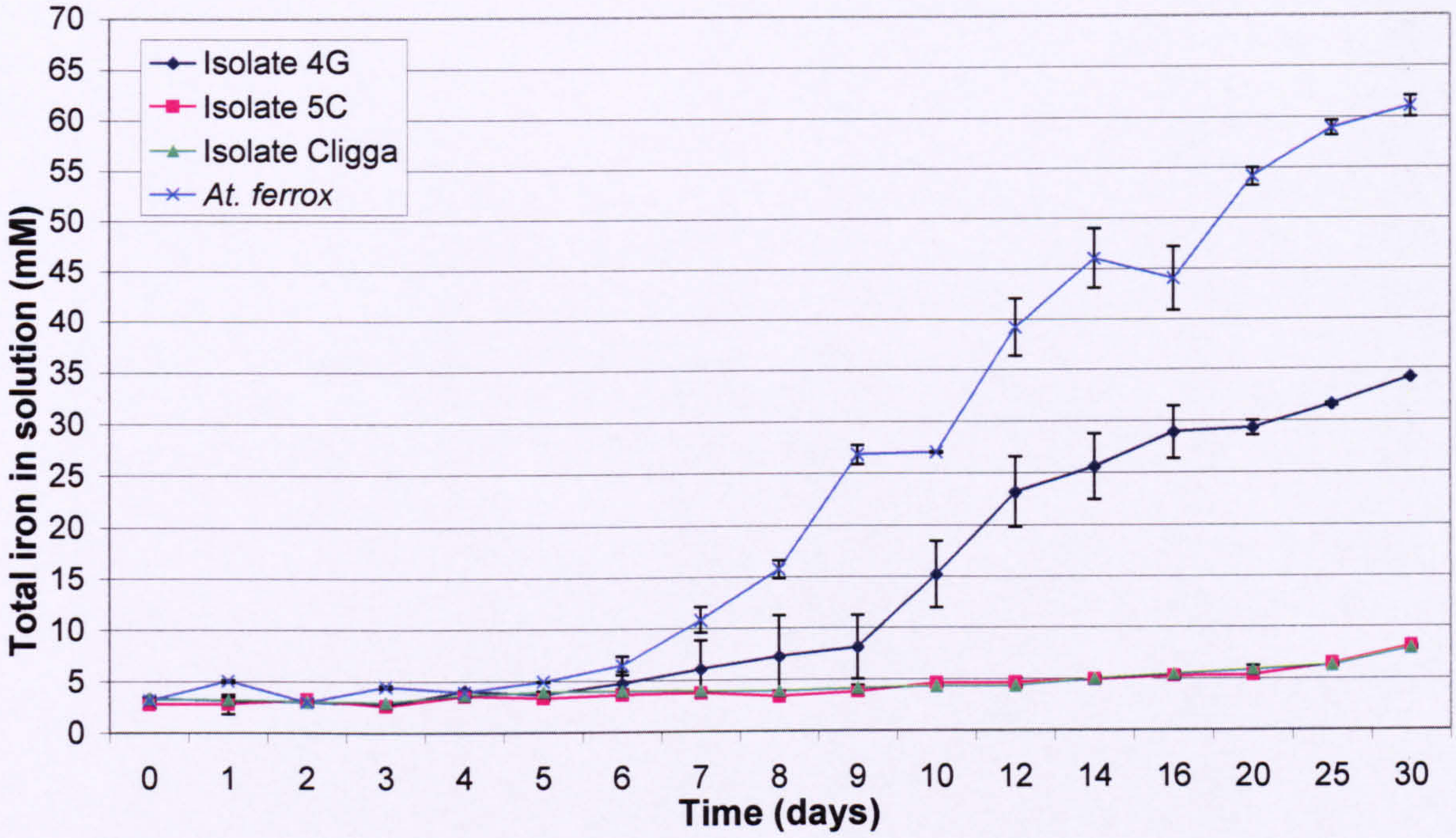


Figure 5.3 Total iron dissolution from Las Cruces chalcopyrite copper ore by isolated bacteria in medium with 30 gl^{-1} sea salt and *At. ferrooxidans* with no added salt for comparison. Each datum point represents the mean ± standard deviation of duplicate cultures.

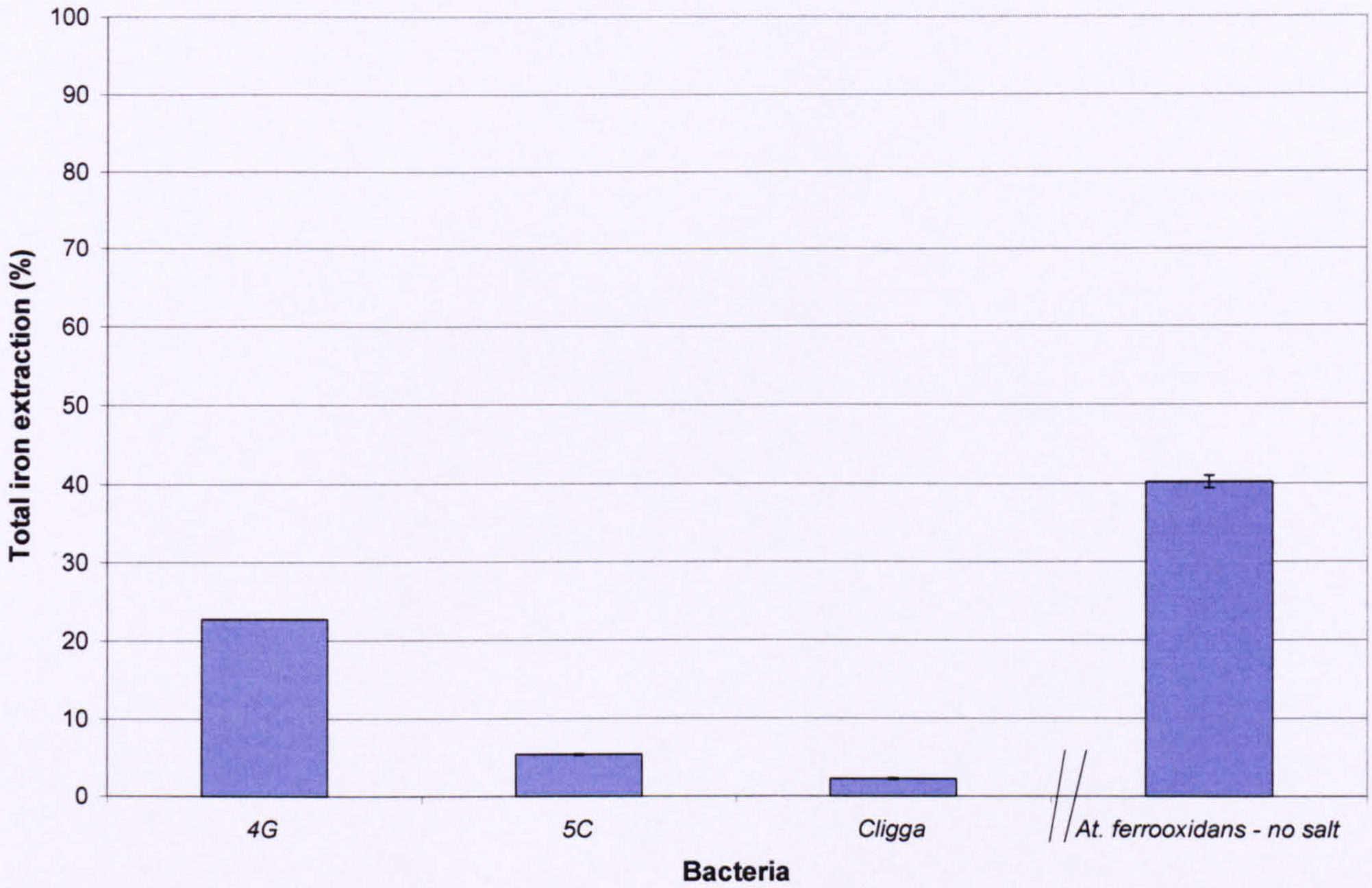


Figure 5.4 Total iron extracted from Las Cruces copper ore by isolated bacteria and *At. ferrooxidans* as a percentage of total iron in ore sample, after 30 days. Each datum point represents the mean ± standard deviation of duplicate cultures.

Table 5.1 Average and fastest rates of iron dissolution from Las Cruces copper ore by isolated bacteria *and At. ferrooxidans*

Experiment	Average rate of iron dissolution (mM day^{-1})	Fastest rate of iron dissolution (mM day^{-1})
4G	1.03	2.97
5C	0.18	0.23
Cligga	0.15	0.16
At. ferrooxidans	2.01	6.78

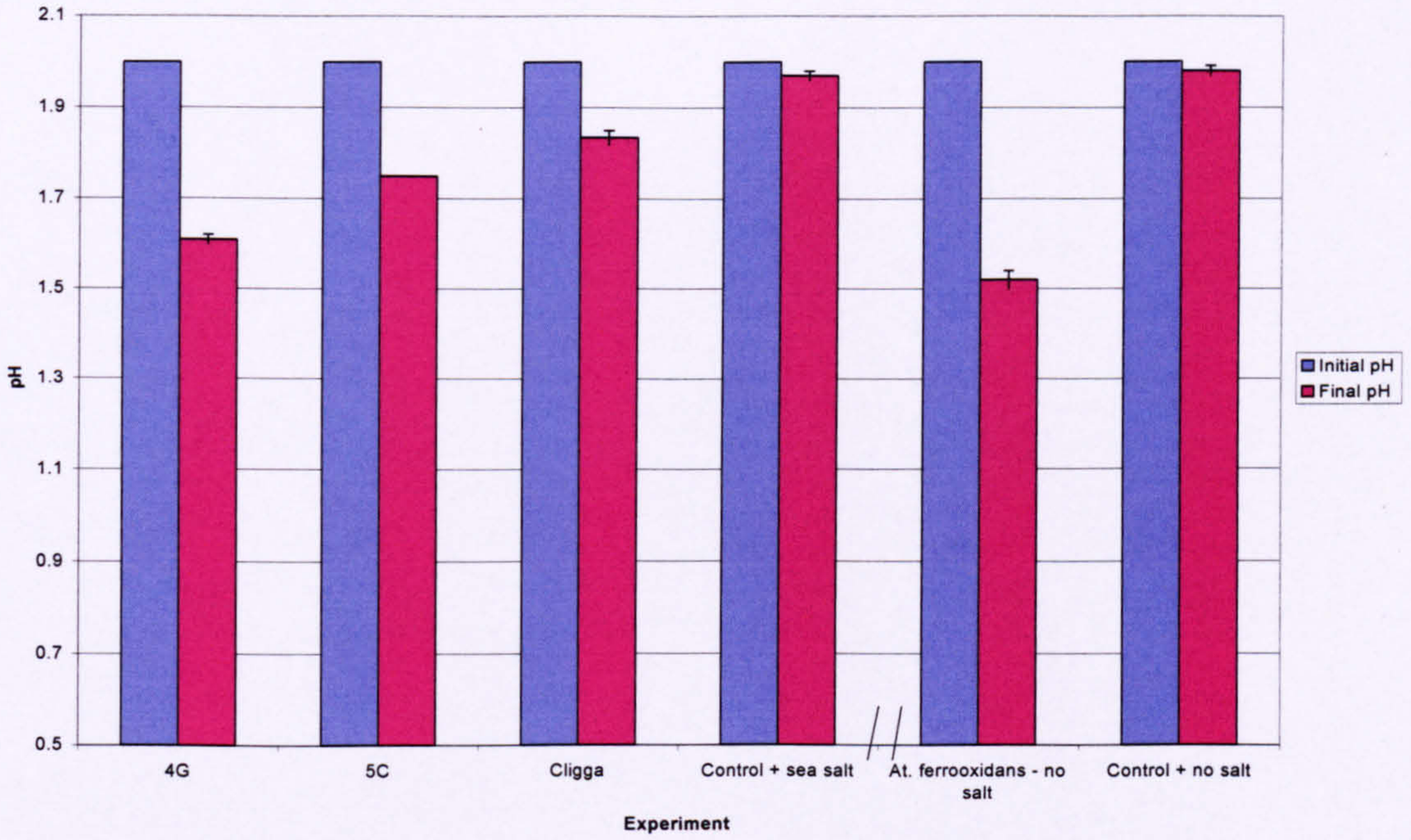


Figure 5.5 Initial and final pH of Las Cruces copper ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

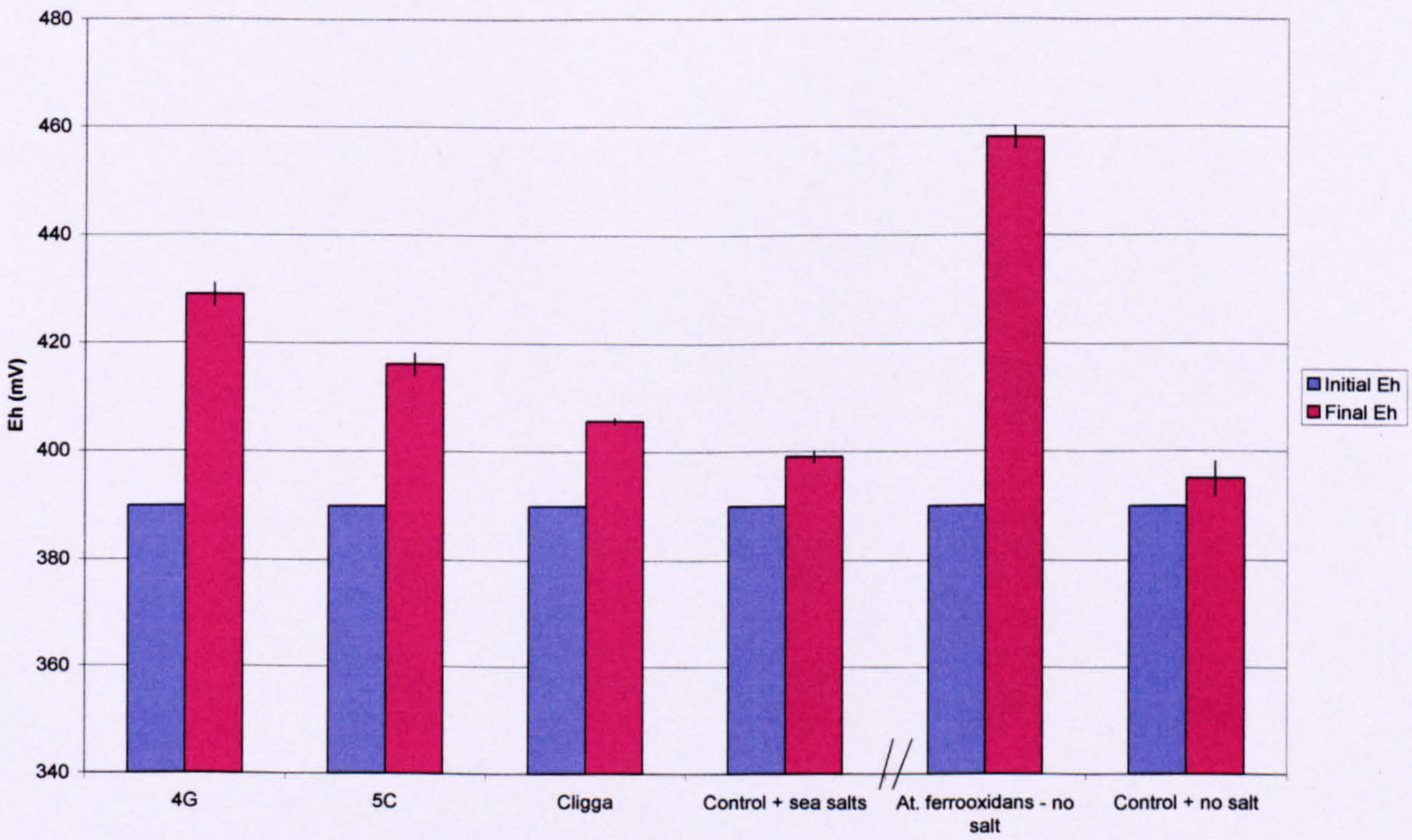


Figure 5.6 Initial and final redox potential of Las Cruces copper ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

5.3 Bioleaching of Lihir gold by isolated bacteria

5.3.1 Introduction

The gold mine on Lihir Island, Papua New Guinea currently operates a whole-ore pressure leach system to decompose the sulphide in high-grade gold ore, prior to cyanidation (Blake et al, 1994) or a carbon-in-leach recovery process. However, Lihir also produces a high level of low-grade gold ore (gold extraction is more difficult from low-grade ore) which is being stockpiled and will be treated when current mining finishes and/or economics dictates.

Low-grade ore is difficult to treat with existing chemical and physical methods, as the gold is generally inaccessible, being complexed within the pyrite and arsenopyrite minerals. Therefore, because this ore is recalcitrant, it cannot be solubilised by the usual process of cyanidation (Rawlings & Silver, 1995 and Rawlings & Woods, 1995). This is where pre-treatment with biooxidation processes would be of high utility, being generally more economic than other methods and generally being less invasive to the surrounding environment. Pre-treatment with bacteria capable of oxidising sulphide phases may uncover the gold or alter the structure of the ore, leaving it susceptible to further conventional extraction methods.

The Lihir mine is a coastal mine site and fresh water is a scarce commodity with high capital costs. It would therefore be of great economic and environmental significance if seawater could be used as a lixivant in any prospective bioleaching processes. The top of the Lihir caldera is only about 50 metres above sea level and the projected final depth of the mine is 185 metres below sea level, therefore the logistics of transporting seawater to the mine site or ore to sea level are uncomplicated. However, sea water is toxic to the traditional bacterial species used in bioleaching processes (Cameron *et al*, 1984) and therefore halotolerant iron-oxidising bacteria, such as those described in this study, would be of high utility in such processes, if a process rate advantage could be demonstrated.

5.3.2 Growth and iron dissolution kinetics of the isolated bacteria when grown on Lihir gold ore

The isolated bacteria exhibited extremely short lag phases before exponential growth occurs on Lihir ore, and they all reached stationary phase around day three (Figure 5.7). The exponential growth rate constants were as follows; 4G 1.56 day^{-1}, 5C 1.59 day^{-1}, and Cligga 1.45 day^{-1}. These are very good growth rate constants for these types of bacteria and consequently generation times were all under 17 hours (15.36,15.12 and 16.56 hours respectively).

Ferrous iron concentrations in Lihir cultures of the isolated bacteria (Figure 5.8), decreased from the beginning of the experiment until day three, in correlation with the growth of the bacteria, which may have been using up the ferrous iron to provide energy for growth. Ferrous iron then increased until the end of the experiment in concert with an increase in the levels of total iron dissolution. All of the isolates exhibited high extraction of iron from Lihir ore (Figure 5.9). The total iron extracted by day thirty of the experiment was as follows; 4G 0.96 gl^{-1}, 5C 1.59 gl^{-1}, Cligga 1.29 gl^{-1} (in the presence of 30 gl^{-1} sea salts) and 1.59 gl^{-1} by *At. ferrooxidans* (when grown in the absence of sea salts). These values were a high percentage of the total iron available as can be seen in Figure 5.10. Strain 5C and *At. ferrooxidans* extracted 100 % of the total iron into solution. Strain and 4G and Cligga extracted 66.10 % and 88.86 % of the total iron respectively. All the test bacteria had correspondingly high rates of iron oxidation as shown in Table 5.2.

The change in pH in the Lihir ore cultures is shown in Figure 5.11. It can be seen that the greatest drop in pH occurred in the 5C culture, which also showed the fastest rate of iron dissolution of those tested, having extracted 100% of the total iron into solution over the test period. Figure 5.12 shows the change in redox potential in the Lihir leaching experiments and all of the cultures of the halotolerant strains showed an increase in Eh above the control value and this increase indicated good leaching conditions. Cultures of *At. ferrooxidans* exhibited a higher increase in redox potential than the other strains. However, it has been noted that redox potential in cultures of Gram positive bacteria (i.e. the halotolerant strains) may be lower than similar cultures of Gram negative bacteria. (Yahya & Johnson, 2002).

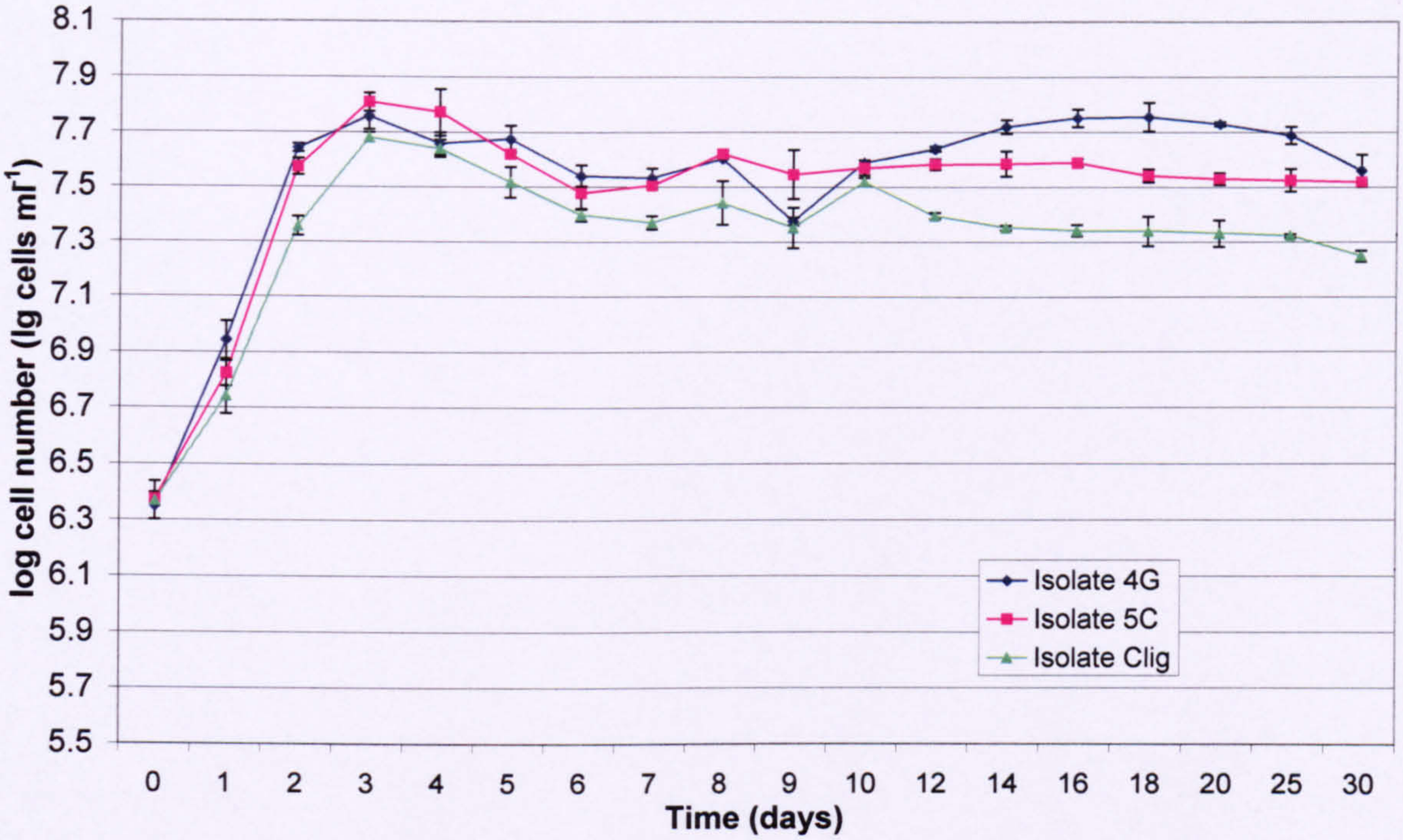


Figure 5.7 Growth of isolated bacteria on 2 % (w/v) Lihir gold ore in medium with 30 gl^{-1} sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

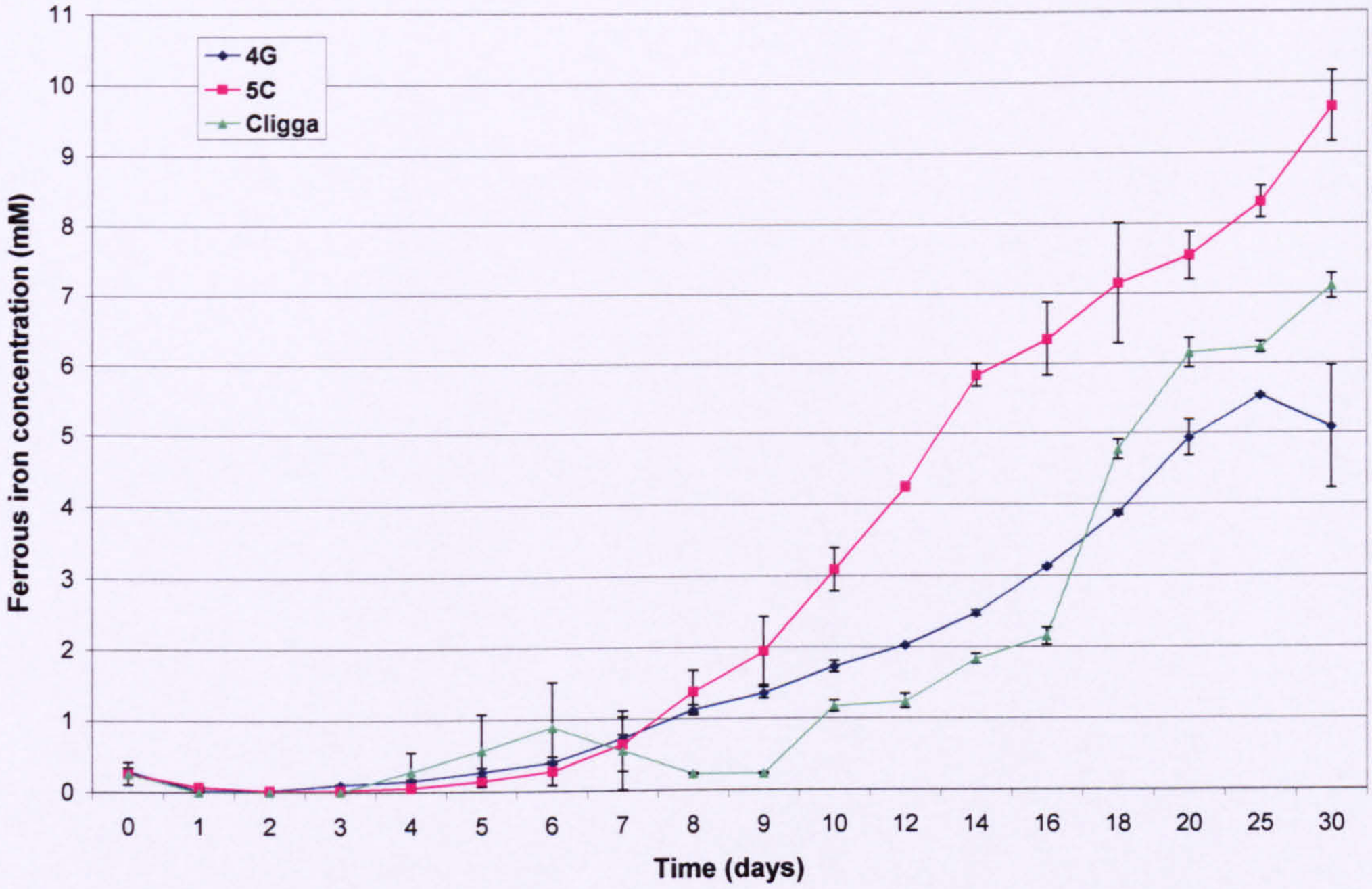


Figure 5.8 Change in ferrous iron concentration in cultures of isolated bacteria grown on Lihir gold ore. Each datum point represents the mean ± standard deviation of duplicate cultures.

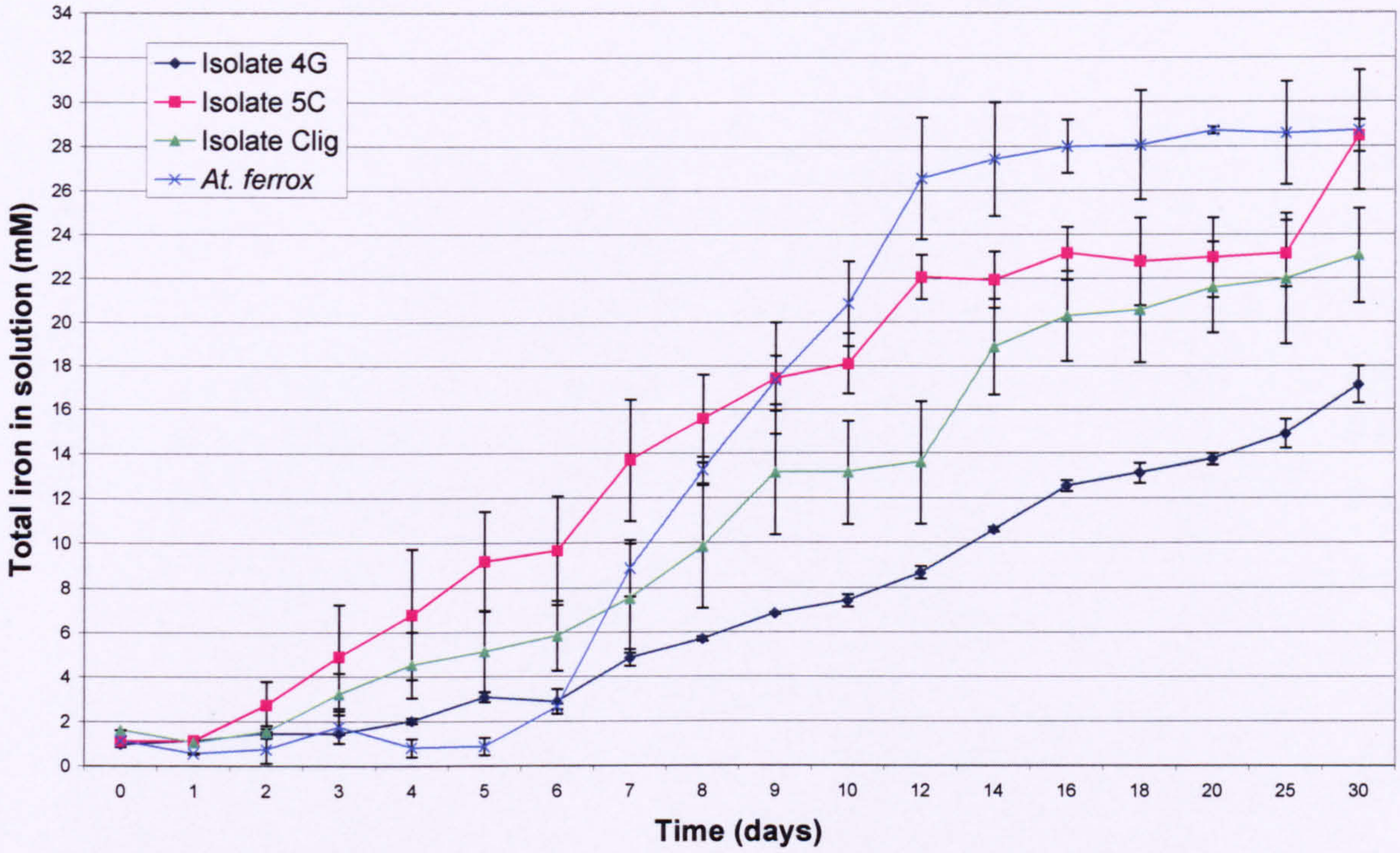


Figure 5.9 Total iron dissolution from Lihir gold ore by isolated bacteria in medium with 30 gl^{-1} added sea salts and *At. ferrooxidans* with no added sea salt. Each datum point represents the mean ± standard deviation of duplicate cultures.

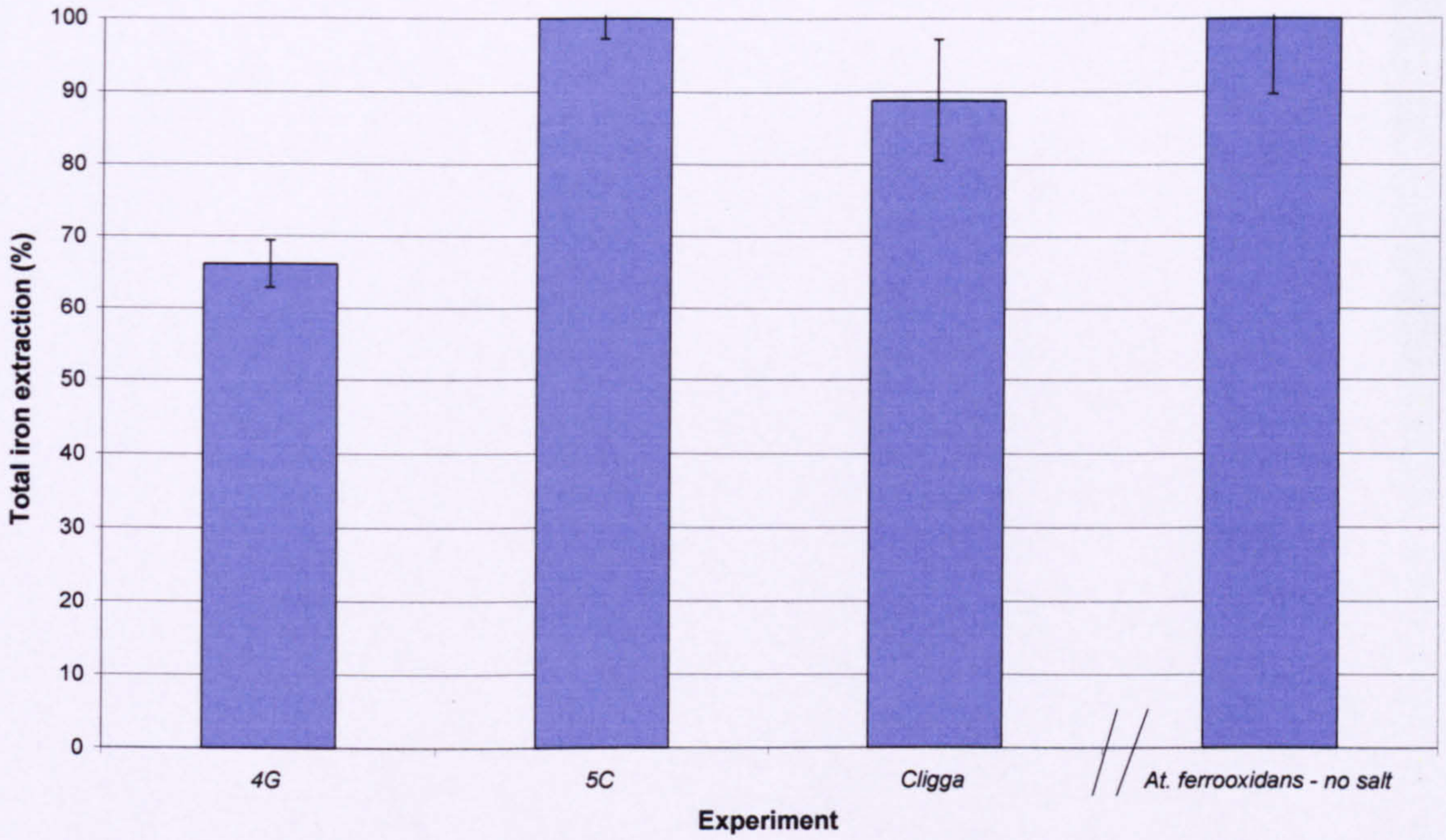


Figure 5.10 Total iron extracted from Lihir gold ore by isolated bacteria and *At. ferrooxidans* as a percentage of total iron in ore sample. Each datum point represents the mean ± standard deviation of duplicate cultures.

Table 5.2 Average and fastest rates of iron dissolution from Lihir gold ore by isolated bacteria *and At. ferrooxidans*

Experiment	Average rate of iron dissolution (mM day^{-1})	Fastest rate of iron dissolution (mM day^{-1})
4G	0.54	0.85
5C	0.91	2.07
Cligga	0.71	1.63
At. ferrooxidans	0.91	2.43

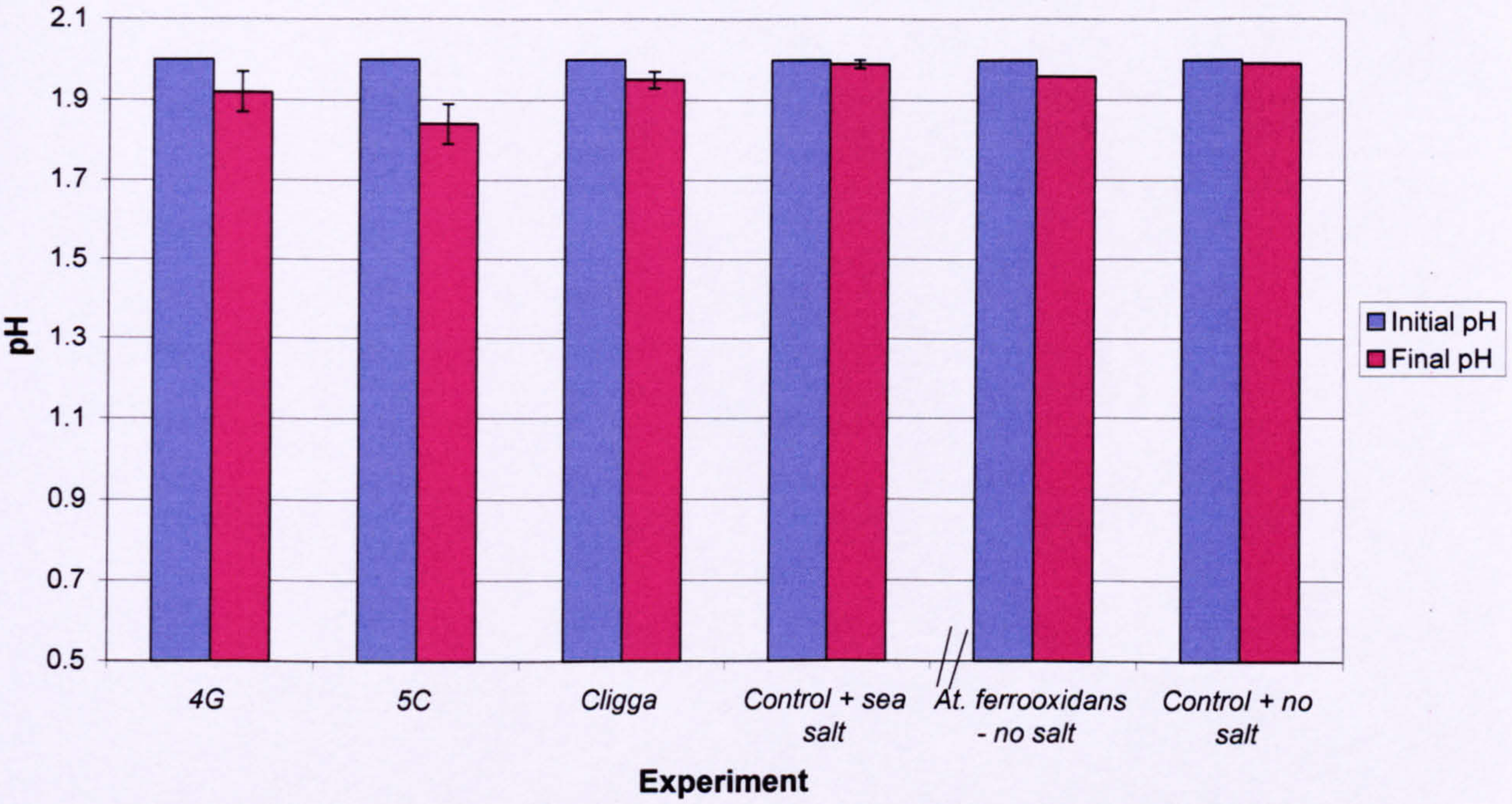


Figure 5.11 Initial and final pH in biooxidation of Lihir gold ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

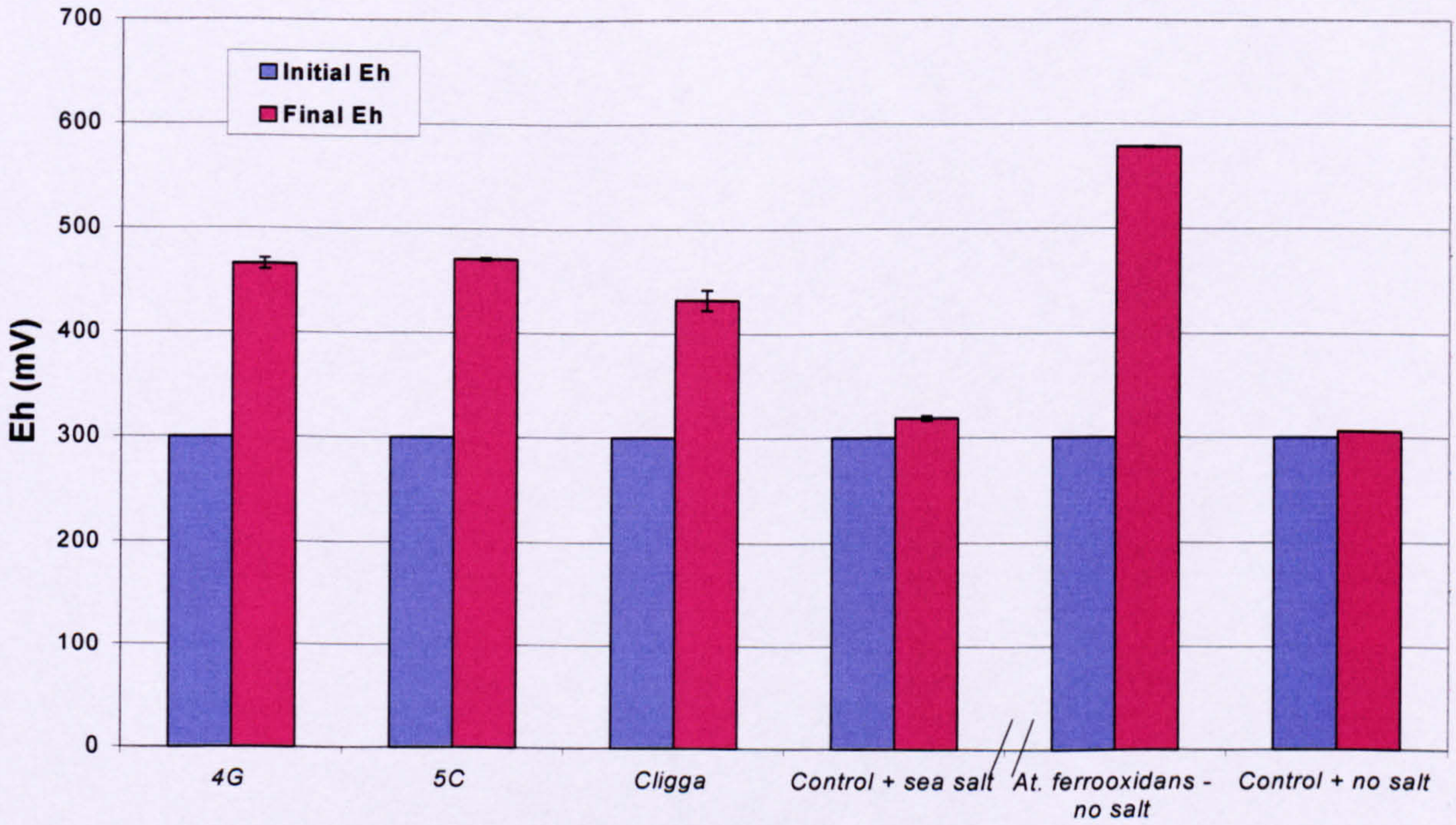


Figure 5.12 Initial and final redox potential in biooxidation of Lihir gold ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

5.4 Bioleaching of the Freeport Rough Feed Ore Sample by isolated bacteria

5.4.1 Introduction

The Freeport ore samples are from the Rio Tinto/Freeport Grasberg operation in Irian Jaya, Indonesia. It is one of the world's largest copper and gold mines in terms of both reserves and production. The ore has a total iron content of 6.65 %, with 2.8 % copper and high amounts of zinc and arsenic (Rio Tinto Technology Ltd; Technical Report).

5.4.2 Growth and iron dissolution kinetics of the isolated bacteria when grown on Freeport Rough Feed copper ore

All of the isolated strains exhibited lag phases of approximately two days, and the exponential phase then lasted until day nine when stationary phase was reached, and cell numbers then decreased from day twenty until the end of the experiment. The exponential growth rate constants of the isolates were relatively slow, being 0.52 day^{-1} for 4G, 0.40 day^{-1} for 5C and 0.49 for Cligga. Consequently generation times were very long, being, 46.08, 60, and 48.96 hours respectively.

Ferrous iron levels in the 5C and Cligga cultures decreased slightly from the beginning to the end of the experiment, with a larger decrease being observed in 4G cultures (Figure 5.14). Total iron dissolution in test cultures (Figure 5.15) generally increased from the beginning to the end of the experiment with a total concentration of iron in solution of 0.39 gl^{-1} by 4G, 0.19 gl^{-1} by 5C and 0.21 gl^{-1} by Cligga. In the absence of added salts, the benchmark microorganism, *At. ferrroxidans* extracted a higher amount of iron from the ore sample, with 0.59 gl^{-1} in solution by day thirty of the experiment. These final iron concentrations are shown in Figure 5.17 with 4G extracting 29.89 % and *At. ferrooxidans* extracting 45.08 % of the total available iron. Rates of iron dissolution can be seen in Table 5.3.

The pH decrease in the test cultures is presented in Figure 5.17 and it was observed that cultures of strain 4G produce the highest drop in pH after 30 days growth on the Freeport rough feed ore sample. The redox potential of cultures of strain 4G showed the greatest increase of the test halotolerant strains (Figure 5.18). This change in pH

and Eh in cultures of 4G was concurrent with the iron oxidation rate of this strain, which was the highest of the halotolerant strains tested on this ore sample.

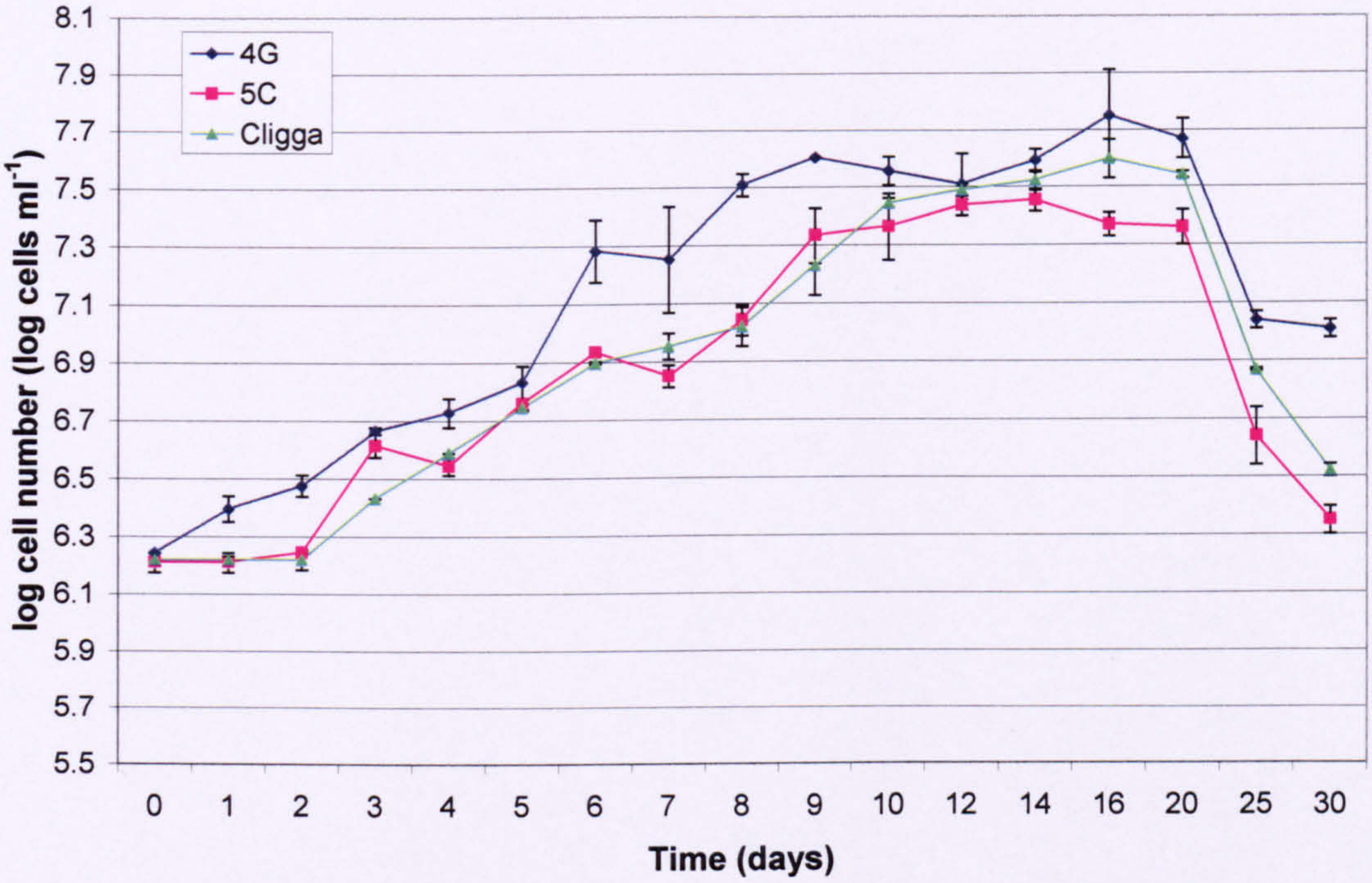


Figure 5.13 Growth of isolated bacteria on 2 % (w/v) Freeport Rough Feed copper ore in medium with 30 gl^{-1} sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

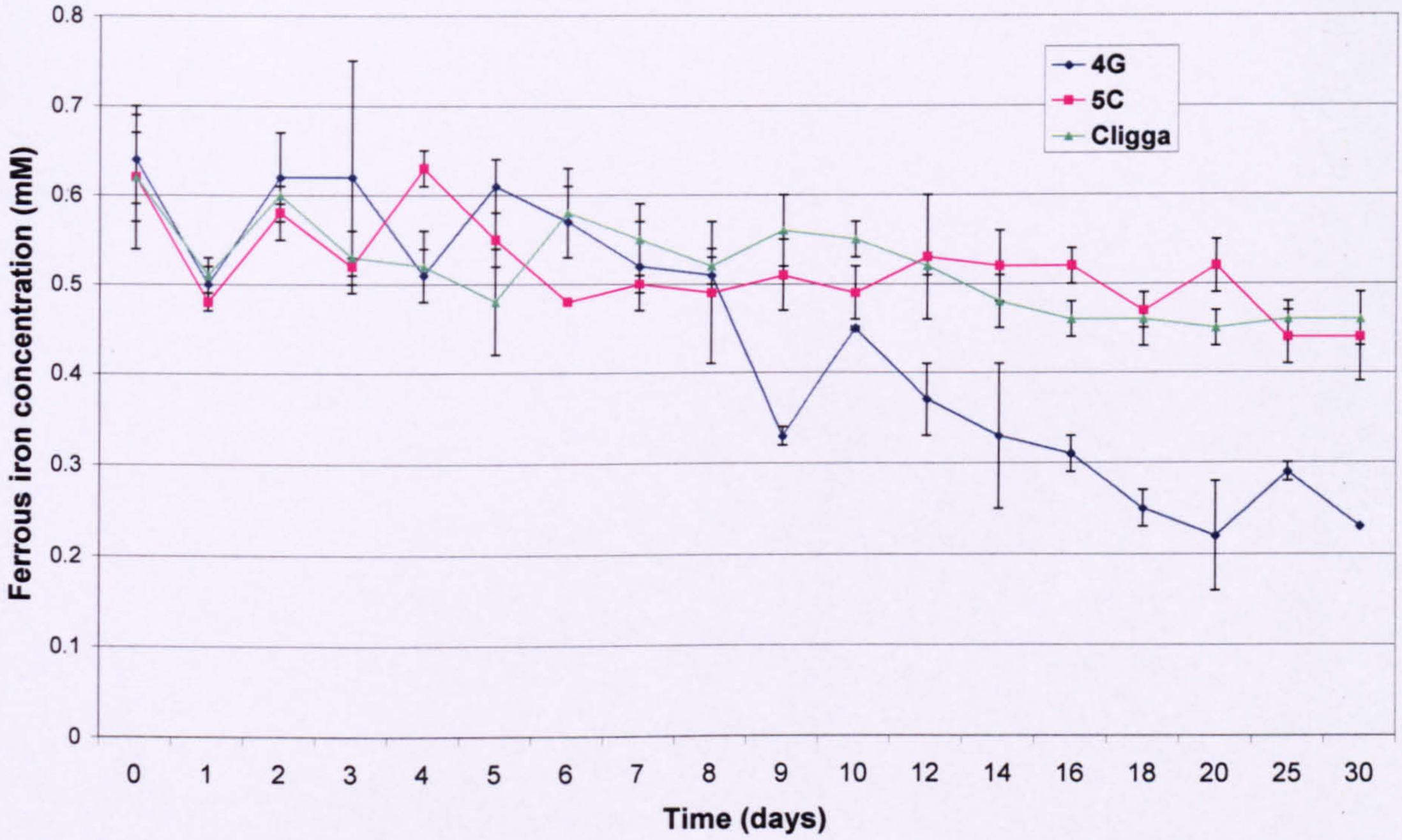


Figure 5.14 Change in ferrous iron concentration in cultures of isolated bacteria grown on Freeport Rough Feed ore sample. Each datum point represents the mean ± standard deviation of duplicate cultures.

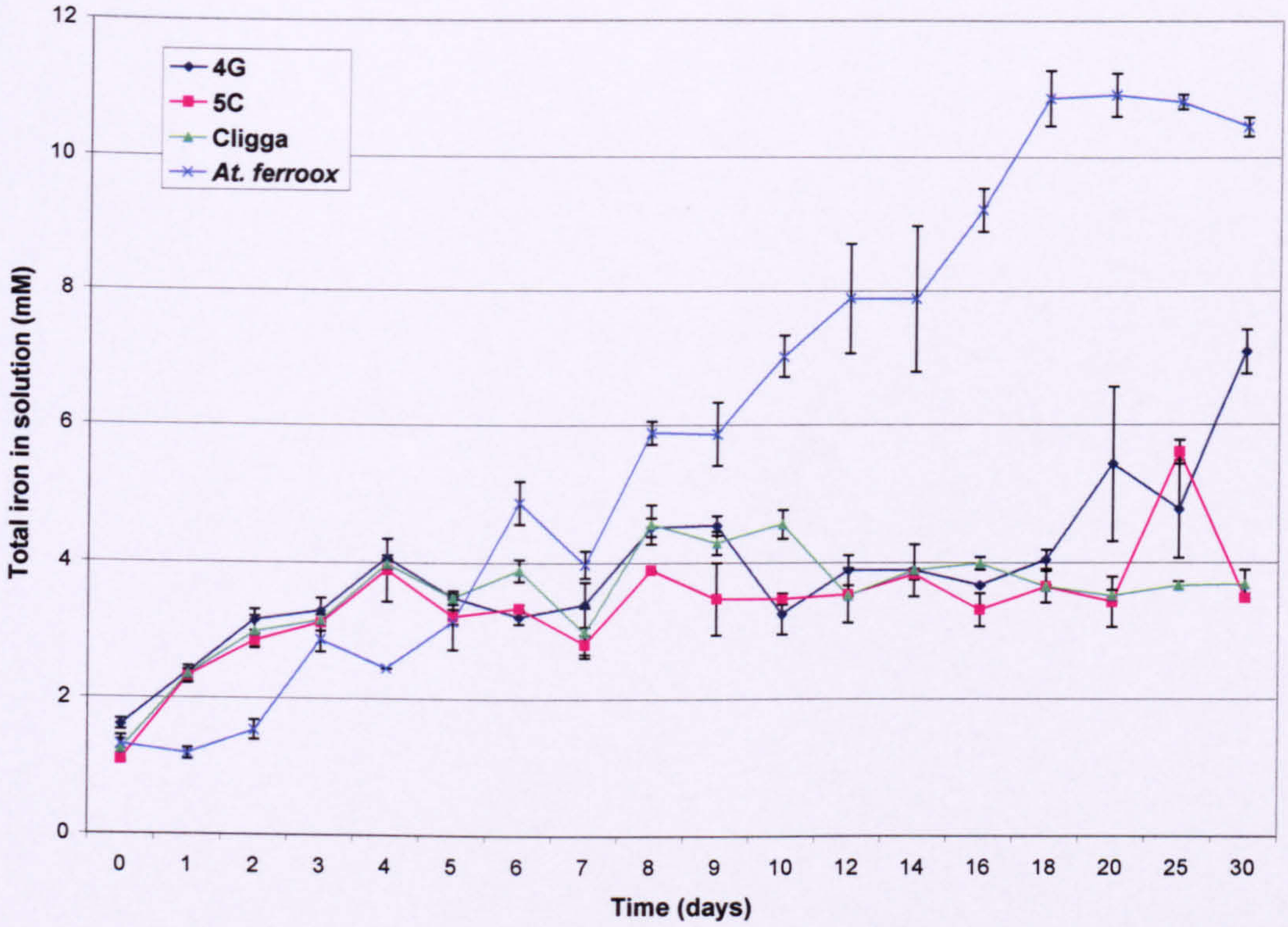


Figure 5.15 Total iron dissolution from Freeport Rough Feed copper ore by isolated bacteria with 30 gl^{-1} sea salt and *At. ferrooxidans* with no added salt. Each datum point represents the mean ± standard deviation of duplicate cultures.

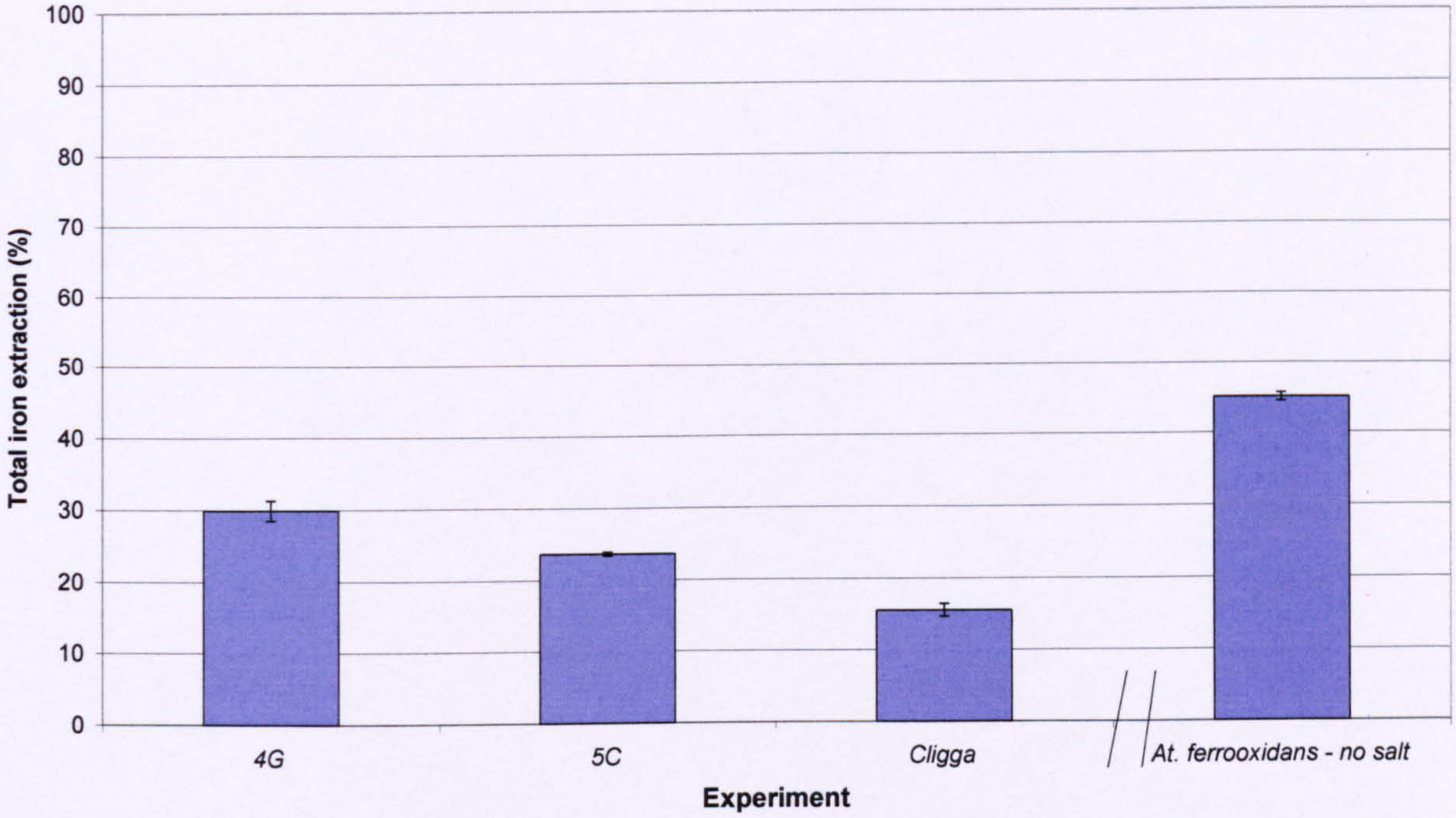


Figure 5.16 Total iron extracted from Freeport Rough Feed copper ore by isolated bacteria and *At. ferrooxidans* as a percentage of total iron in ore sample, after 30 days. Each datum point represents the mean ± standard deviation of duplicate cultures.

Table 5.3 Average and fastest rate of iron dissolution from Freeport Rough Feed copper ore

Experiment	Average rate of iron dissolution (mM day^{-1})	Fastest rate of iron dissolution (mM day^{-1})
4G	0.18	0.62
5C	0.15	0.7
Cligga	0.11	0.68
At. ferrooxidans	0.36	0.67

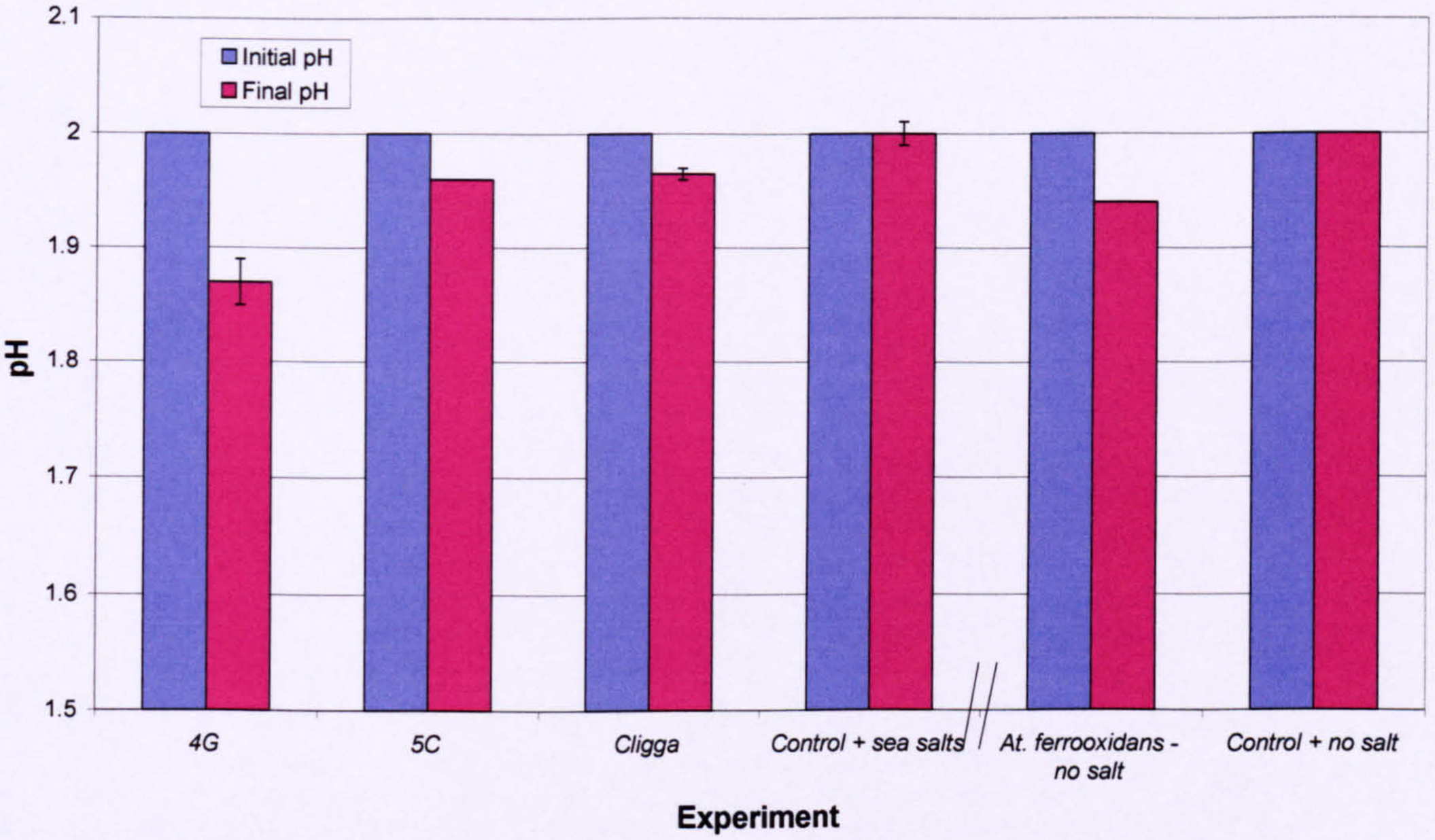


Figure 5.17 Initial and final pH of Freeport Rough Feed ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

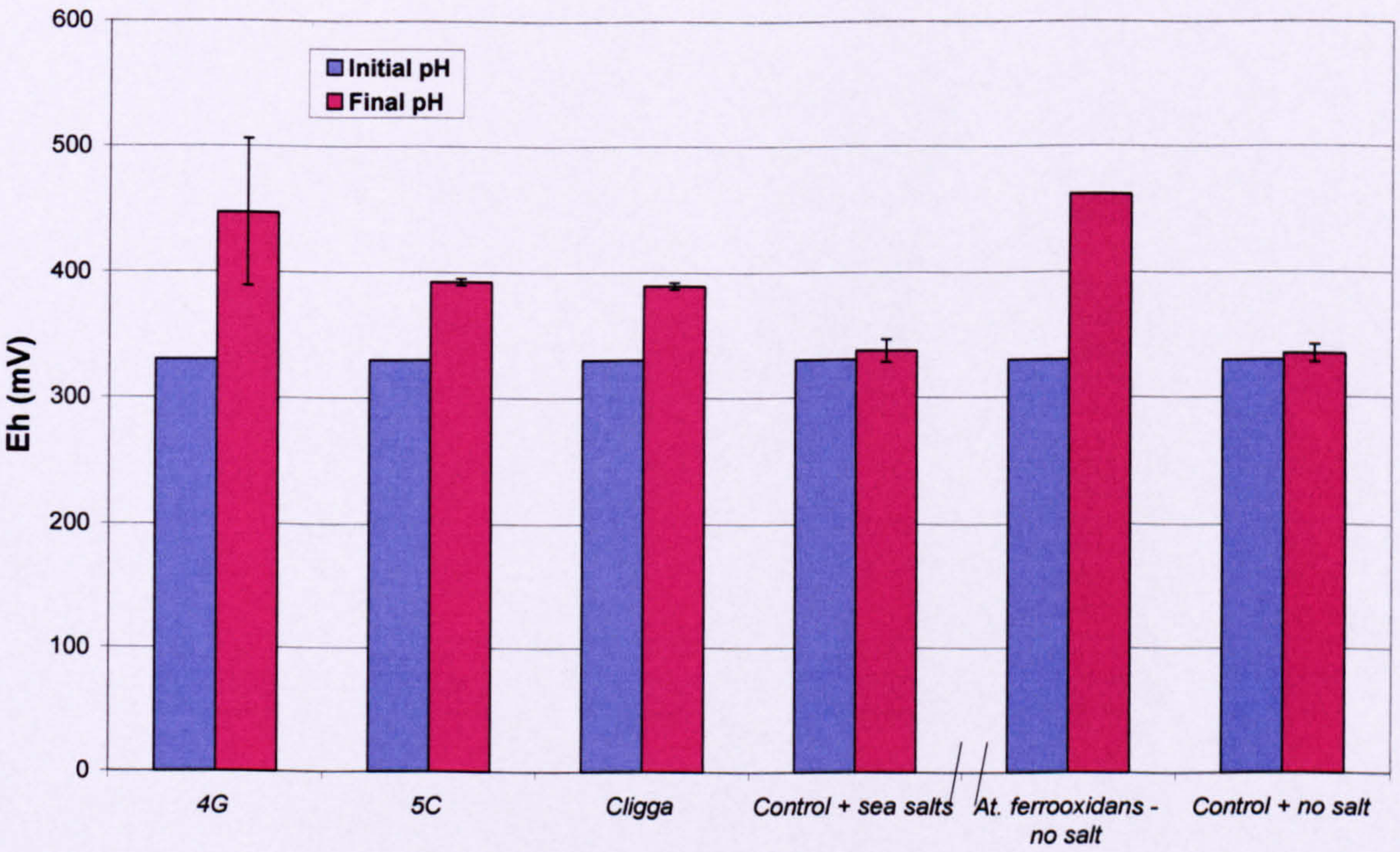


Figure 5.18 Initial and final redox potential of Freeport Rough feed ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

5.5 Bioleaching of Freeport Final Concentrate by isolated bacteria

5.5.1 Introduction

Freeport Final Concentrate is a concentrated version of Freeport Rough Feed; the target metal has been concentrated by flotation methods. The copper content of this ore is 35.77% as compared to 2.8% in the Rougher Feed (Rio Tinto Technology Ltd; Technical Report).

5.5.2 Growth and iron dissolution kinetics of the isolated bacteria when grown on Freeport Final concentrate gold ore

Growth of the isolated bacteria on Freeport Final concentrate was slower than on the previous ore samples (Figure 5.20). The bacteria exhibited a lag phase of around two days and the log phases lasted until approximately day nine, however growth rate constants were rather low. The growth rate constants were; 4G 0.5 day^{-1}, 5C 0.33 day^{-1} and Cligga 0.32 day^{-1}. Therefore, the mean generation times were rather long; 4G 48, 5C 72.72, and Cligga 75 hours.

The concentration of ferrous iron in all of the cultures decreased from the beginning of the experiments until it was all oxidised by the growth of the bacteria (Figure 5.20). Similarly the amount of iron in solution (Figure 5.21) decreased from the beginning of the experiments, due, as mentioned above, to the oxidation of the ferrous iron in solution. The amount of iron in solution then did not increase for the duration of the experiments, indicating that no oxidation of the metaliferrous ore took place in these cultures. However, the benchmark microorganism, *At. ferrooxidans*, solubilised a total of 0.74 gl^{-1} iron after thirty days of growth on Freeport Final concentrate, which was 14.47 % of the total iron available.

Figure 5.22 shows the change in pH in the test cultures after 30 days growth on the Freeport final concentrate. The pH fell in the test halotolerant cultures even though no iron dissolution was observed in these experiments, however, the bacteria may have initially been growing using the yeast extract and producing H_2SO_4 as a by-product of metabolism as growth was observed in these cultures (Figure 5.19). Redox potential in

the cultures of salt tolerant bacteria showed only a small increase after 30 days growth (Figure 5.23) and this consolidated the results obtained that indicated that there was very little iron dissolution being mediated by these bacteria.

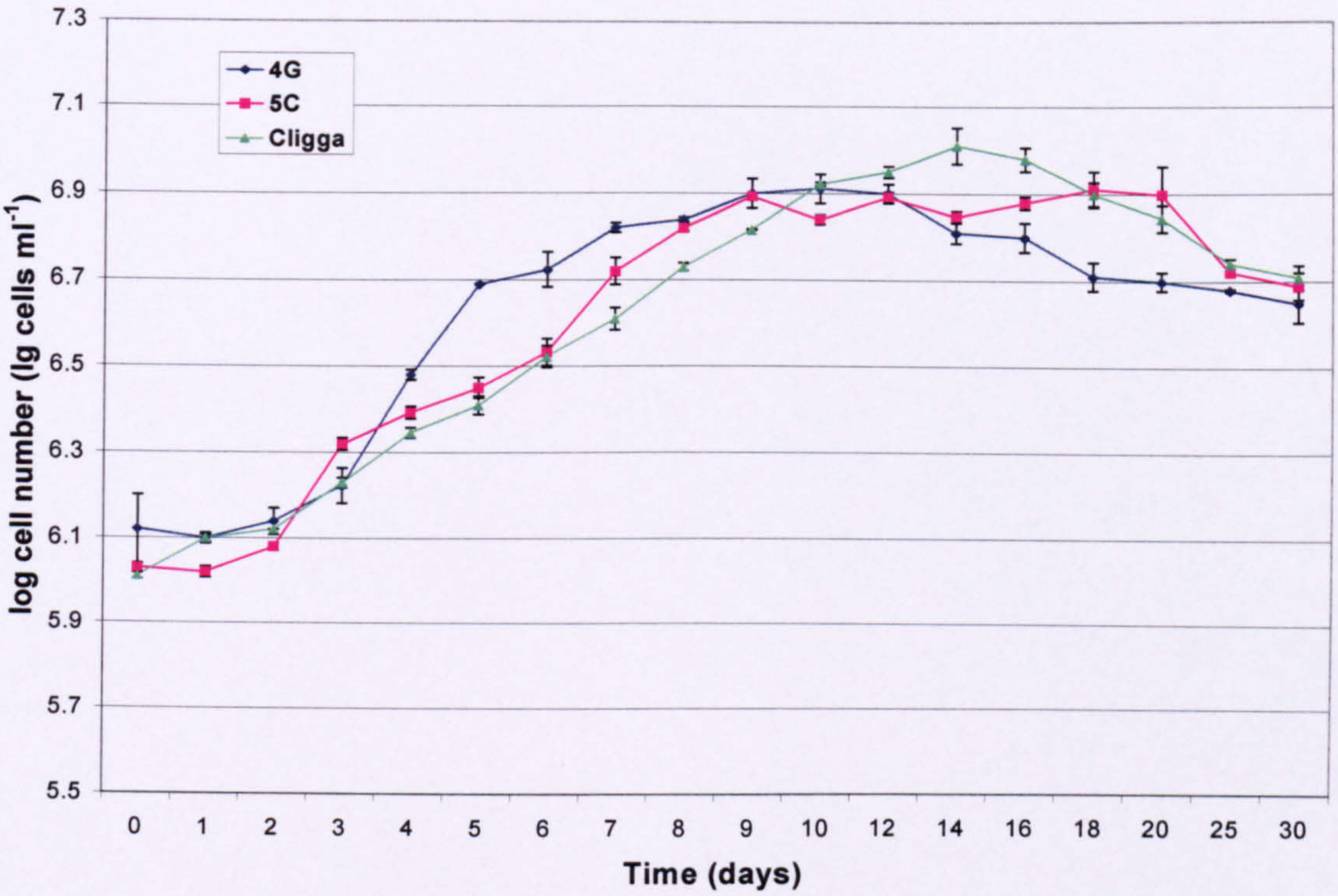


Figure 5.19 Growth of isolated bacteria on 2 % (w/v) Freeport Final concentrate in medium with 30 gl^{-1} sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

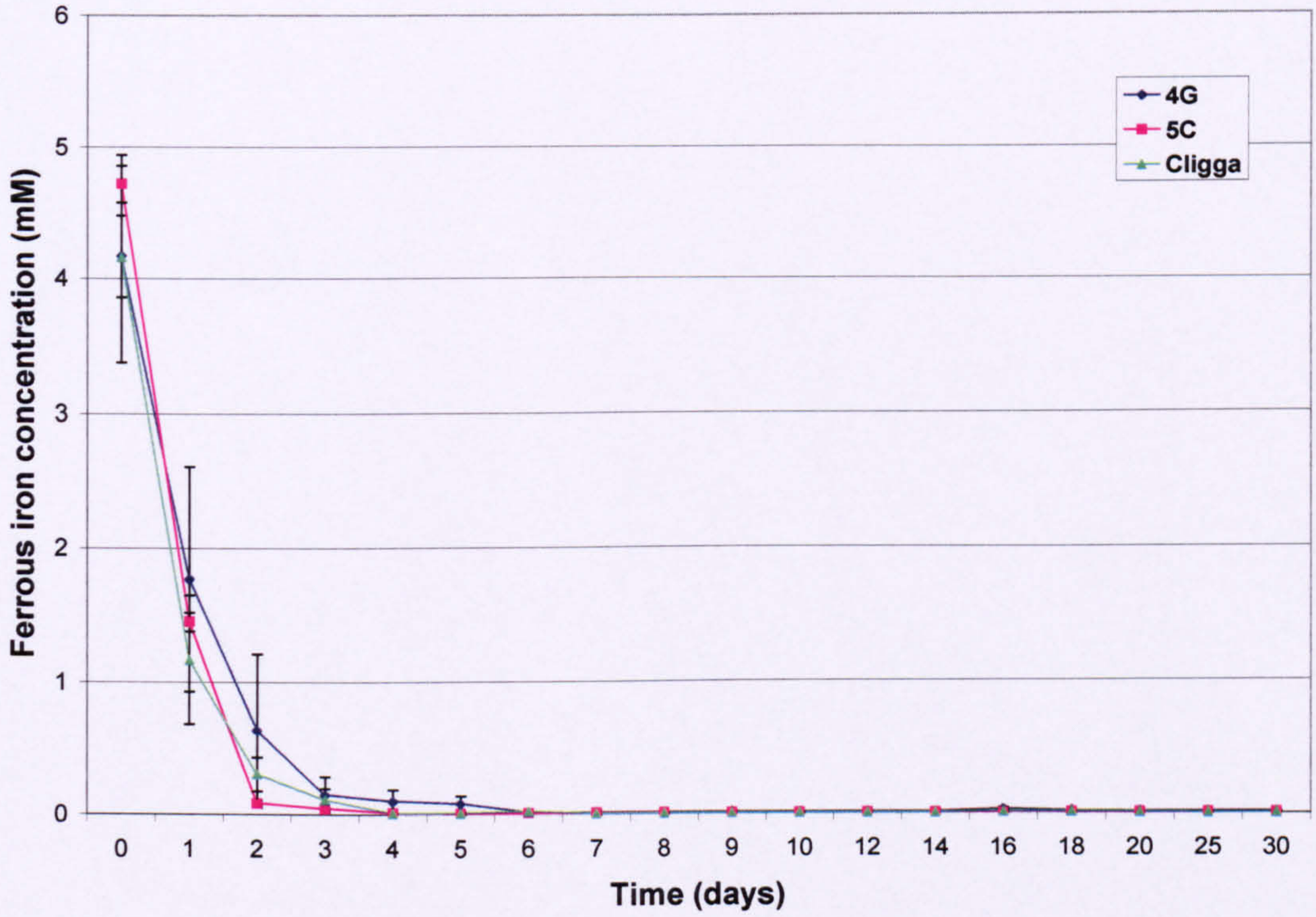


Figure 5.20 Change in ferrous iron concentration in cultures of isolated bacteria grown on Freeport Final concentrate ore sample. Each datum point represents the mean ± standard deviation of duplicate cultures.

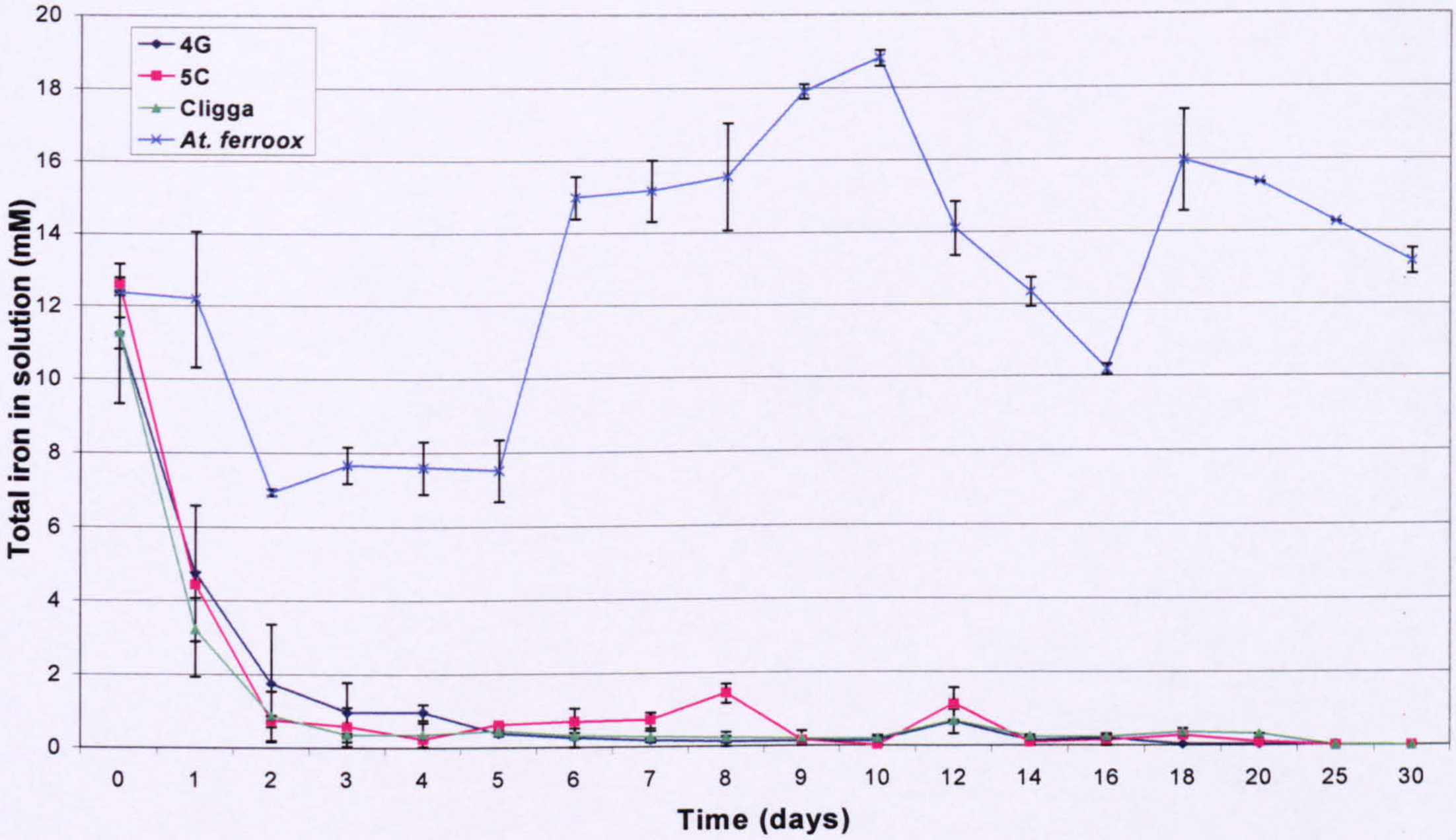


Figure 5.21 Total iron dissolution from Freeport Final Concentrate ore by isolated bacteria with 30 gl^{-1} sea salts and *At. ferroxidans* with no added salt. Each datum point represents the mean ± standard deviation of duplicate cultures.

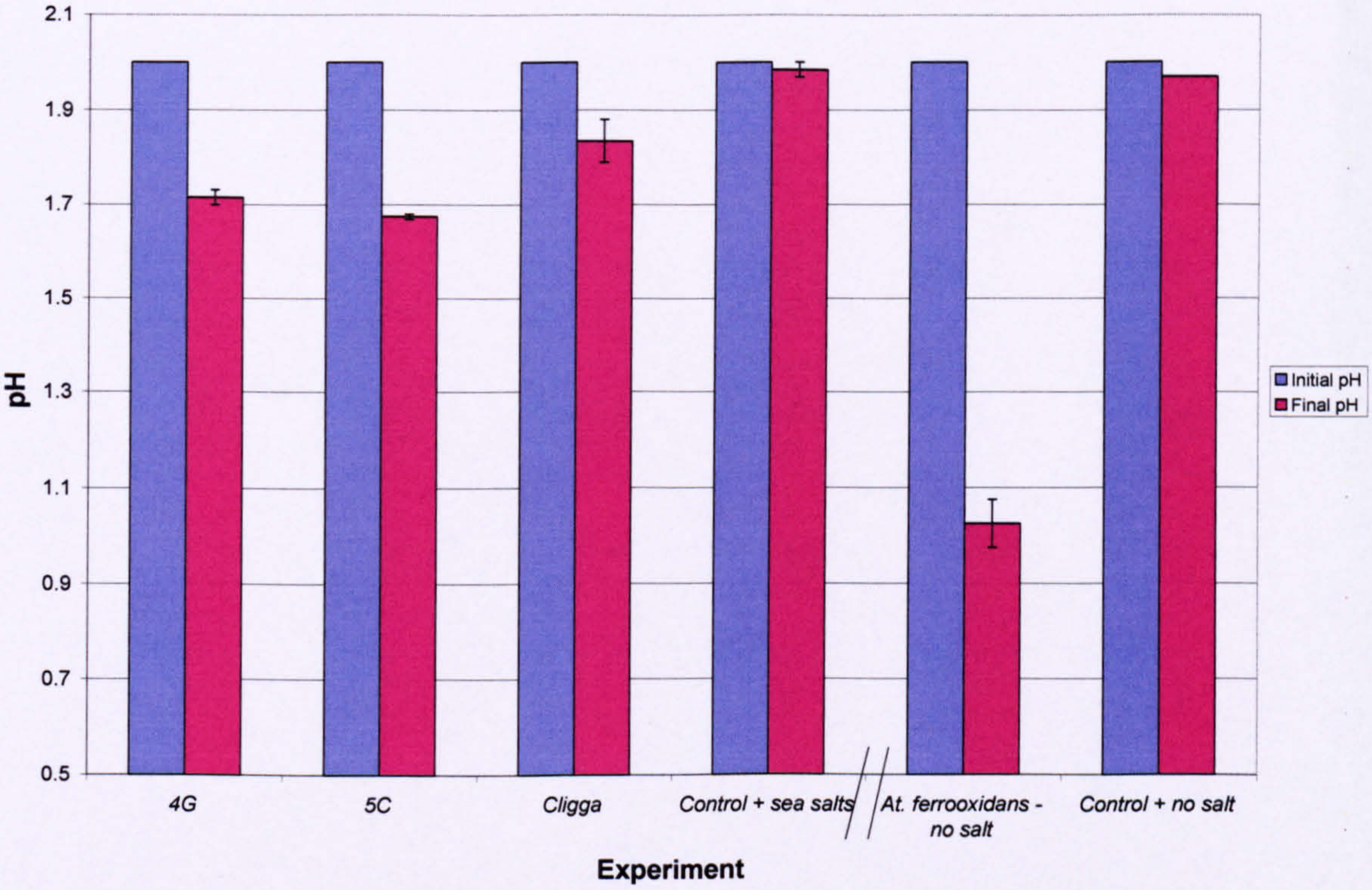


Figure 5.22 Initial and final pH of Freeport Final concentrate experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

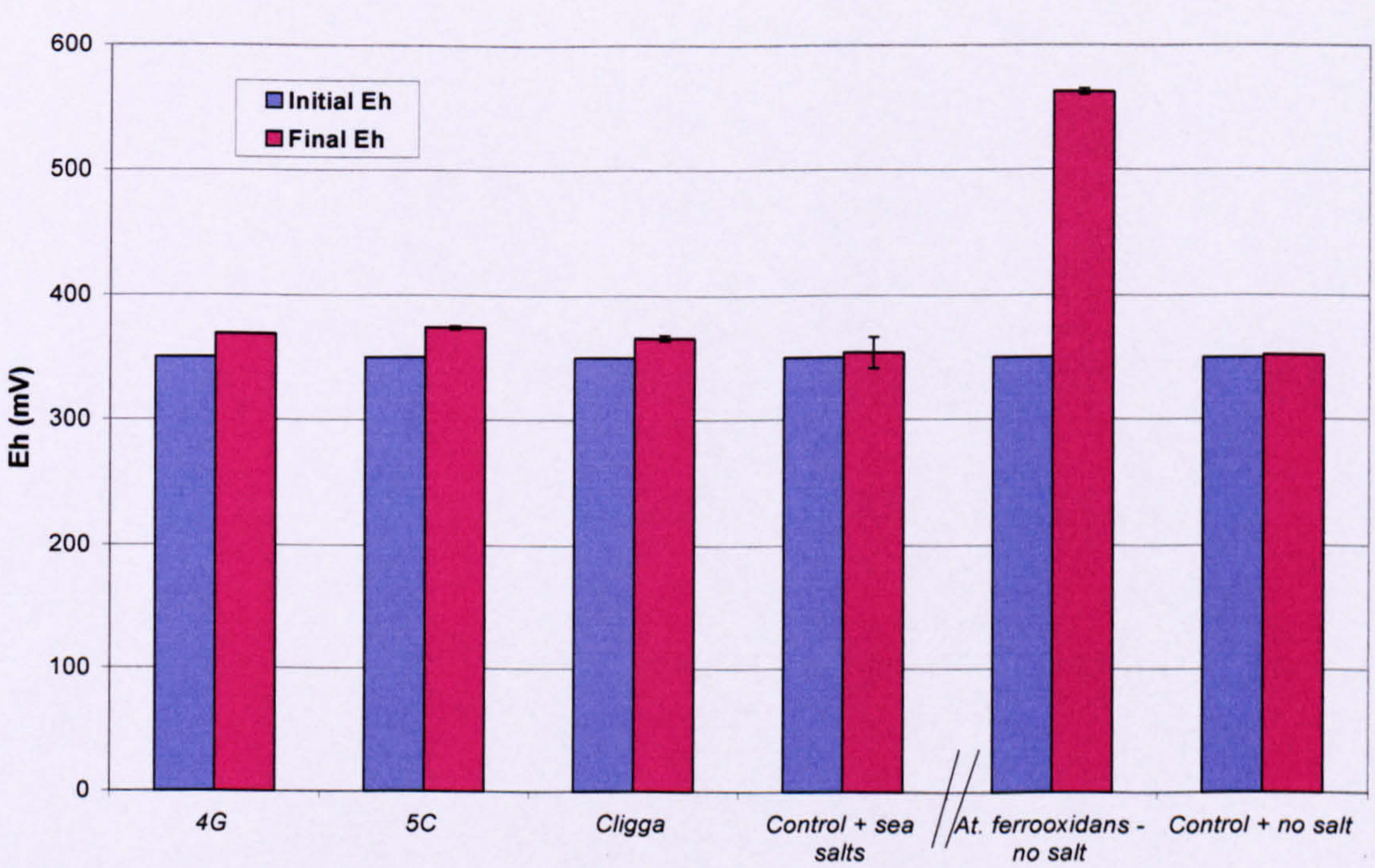


Figure 5.23 Initial and final redox potentials of Freeport Final Concentrate ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

5.6 Bioleaching of Somicor ore sample by the isolated bacteria

5.6.1 Introduction

The Somincor (Sociedade Mineira de Neves Corvo) operation is a high-grade copper mine in the Alenejjo region of Southern Portugal. Production is currently about 100,000 tonnes of copper (in concentrate form) per annum. Tin and silver are also extracted from this mine, though in smaller amounts. The Somincor ore sample is composed of chalcopyrite, gangue pyrite with minor bornite and digenite. This ore has a copper load of 23.76 % and iron comprises 30.89 % of the total mineral (Rio Tinto Technology Ltd; Technical Report).

5.6.2 Growth and iron dissolution kinetics of the isolated bacteria when grown on Somincor copper ore

Good growth was observed with all of the test strains on Somincor copper ore samples (Figure 5.24). The strains exhibited lag phases of around two days, after which the log phase persisted until around day twelve. However, exponential growth rate constants were quite low; 4G 0.38 day^{-1}, 5C 0.31 day^{-1} and Cligga 0.25 day^{-1}. These values translate to long mean generation times, being 63.12, 77.52, and 100 hours respectively.

Ferrous iron concentration increased slightly until day eight then decreased again until the end of the experiment (Figure 5.25), but overall the changes in ferrous iron concentration were relatively low. Total iron dissolution by the three isolated bacteria was relatively low (Figure 5.26), with 12.61 % of the total iron extracted by 4G, 10.31 by % and 11.22 % by strain Cligga. However, *At. ferrooxidans* extracted 51.63 % of the total iron form the ore after thirty days of growth on this ore (Figure 5.27). Table 5.4 shows the rate of iron-oxidation of the test strains.

Figure 5.28 shows the change in pH in cultures of the test bacteria when grown in medium with the Somincor ore sample. This figure shows that the pH has decreased more in all of the test cultures as compared to the controls, indicating the growth and production of H_2SO_4. The change in redox potential shows a slight increase in all of the

test cultures (Figure 5.29) which is concurrent with iron dissolution occurring in these cultures and therefore an increase in the ferric/ferrous iron ratio.

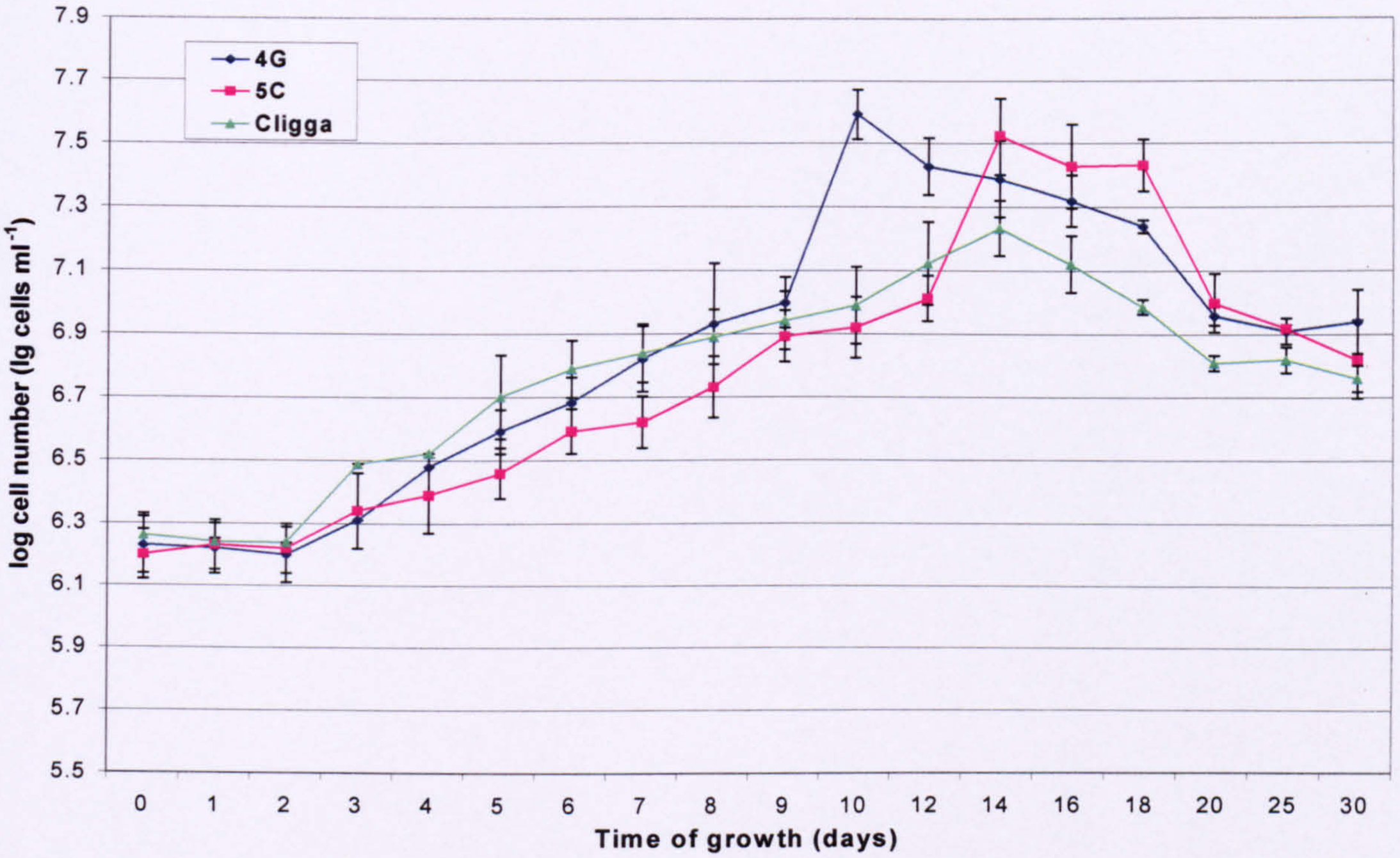


Figure 5.24 Growth of isolated bacteria on 2 % (w/v) Somincor copper ore in medium with 30 gl^{-1} sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

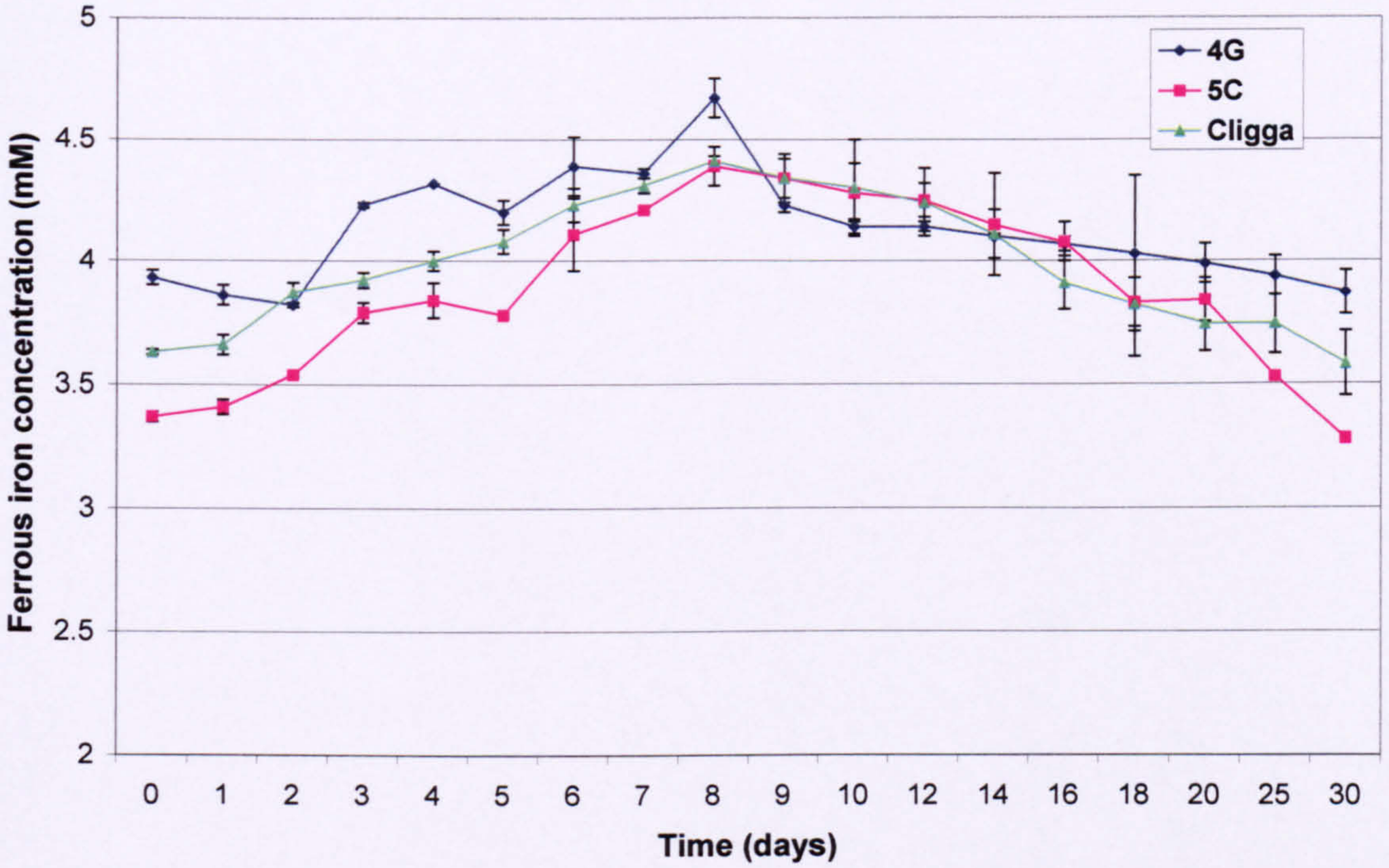


Figure 5.25 Change in ferrous iron concentration in cultures of isolated bacteria grown on Las Cruces chalcopyrite copper ore. Each datum point represents the mean ± standard deviation of duplicate cultures.

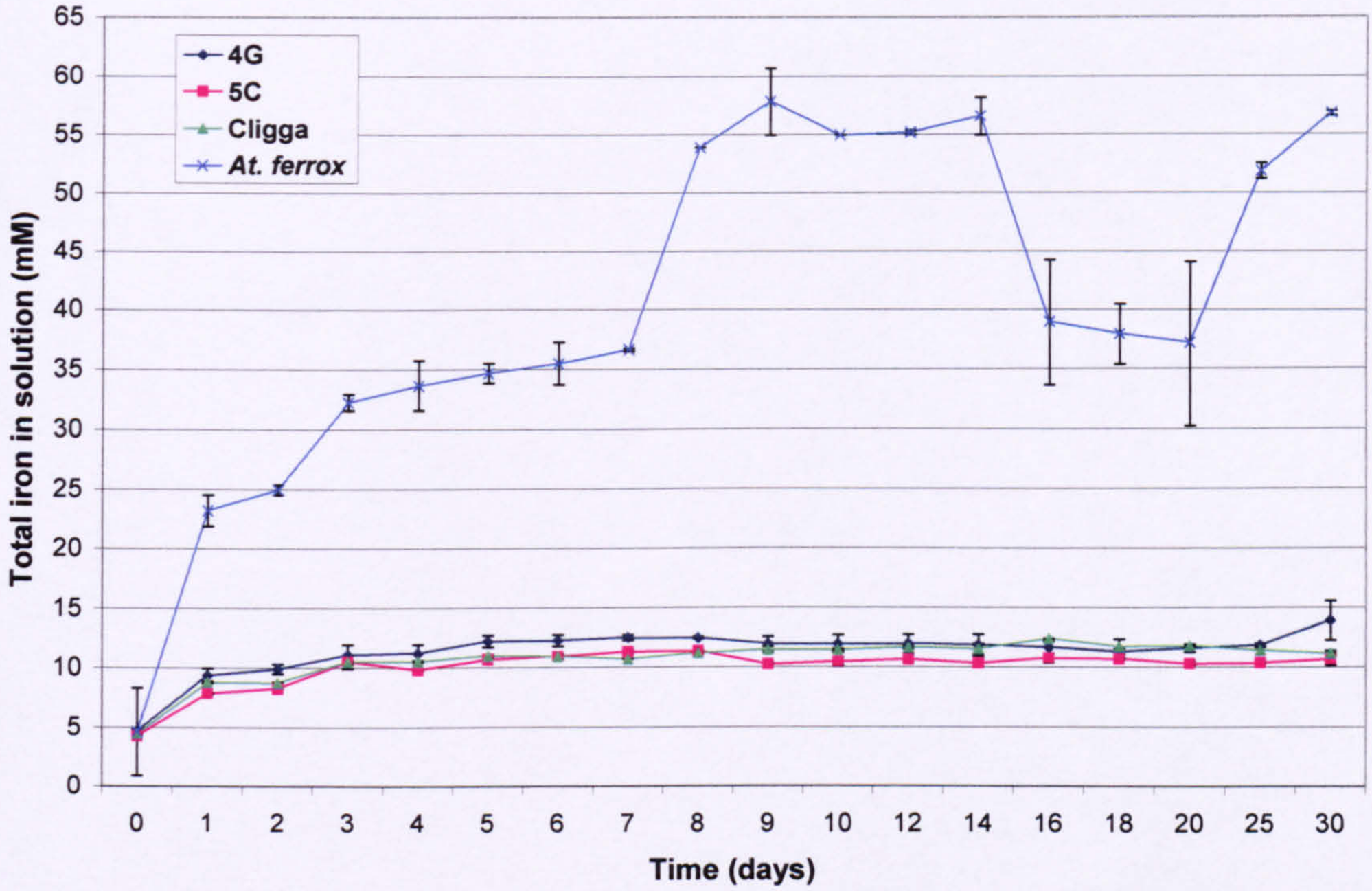


Figure 5.26 Total iron dissolution from Somincor copper ore by isolated bacteria with 30 gl^{-1} sea salts and *At. ferrooxidans* with no added salt. Each datum point represents the mean ± standard deviation of duplicate cultures.

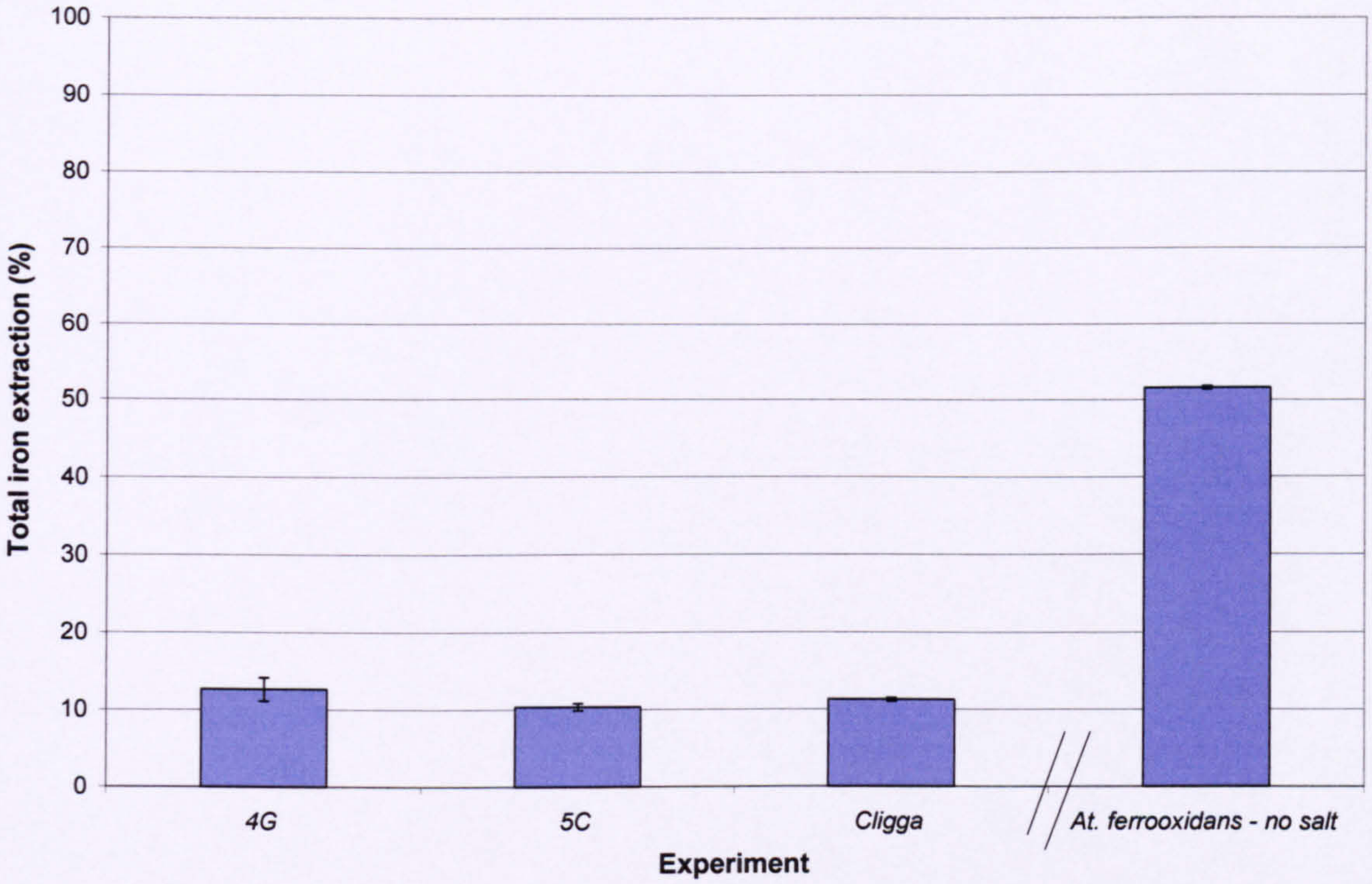


Figure 5.27 Total iron extracted from Somincor copper ore by isolated bacteria and *At. ferrooxidans* as a percentage of total iron in ore sample, after 30 days. Each datum point represents the mean ± standard deviation of duplicate cultures.

Table 5.4 Average and fastest rates of iron dissolution from Somincor copper ore by isolated bacteria *and At. ferrooxidans*

Experiment	Average rate of iron dissolution (mM day^{-1})	Fastest rate of iron dissolution (mM day^{-1})
4G	0.31	1.49
5C	0.24	1.28
Cligga	0.26	1.29
At. ferrooxidans	1.89	10.67

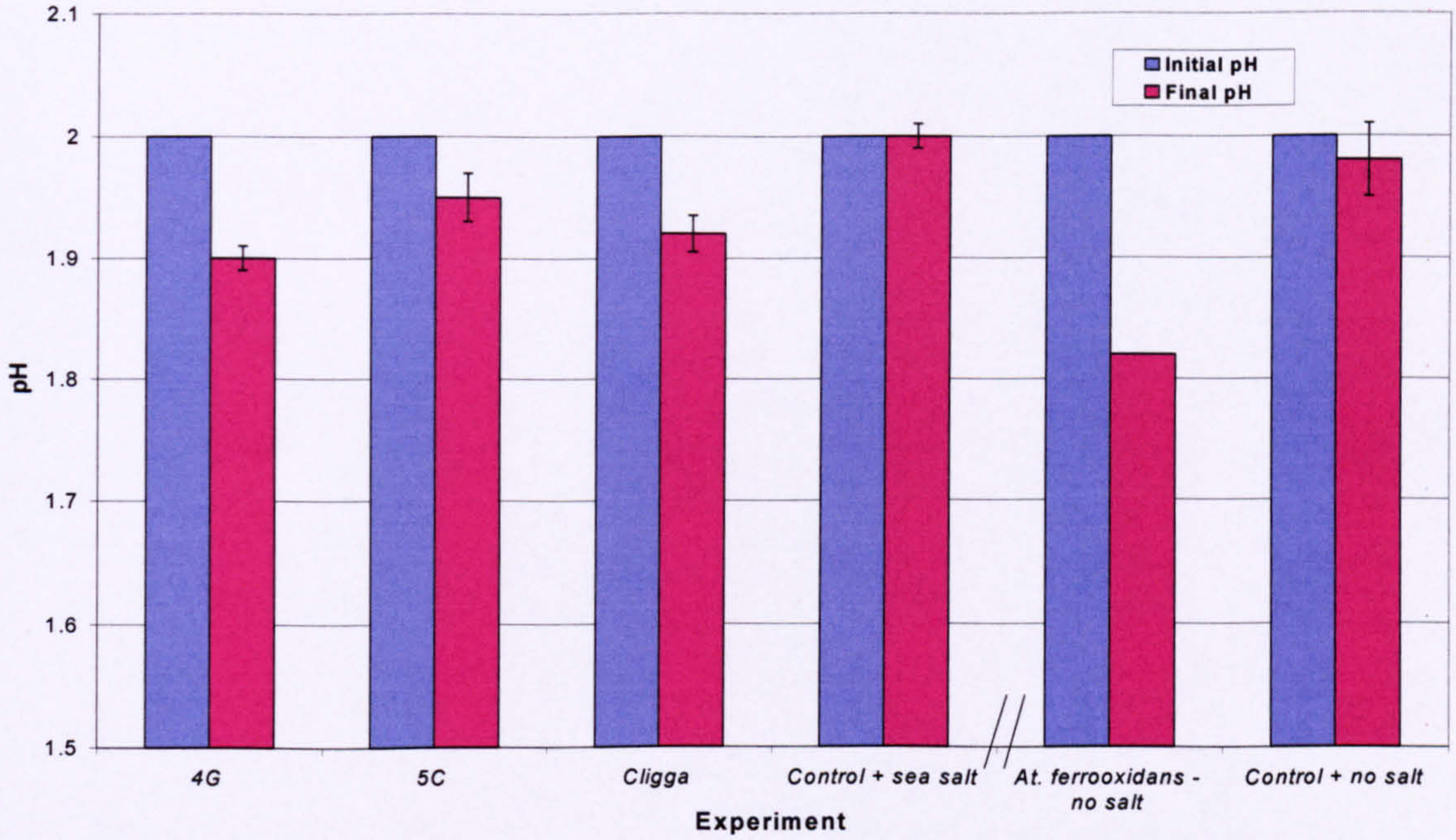


Figure 5.28 Initial and final pH of Somincor copper ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

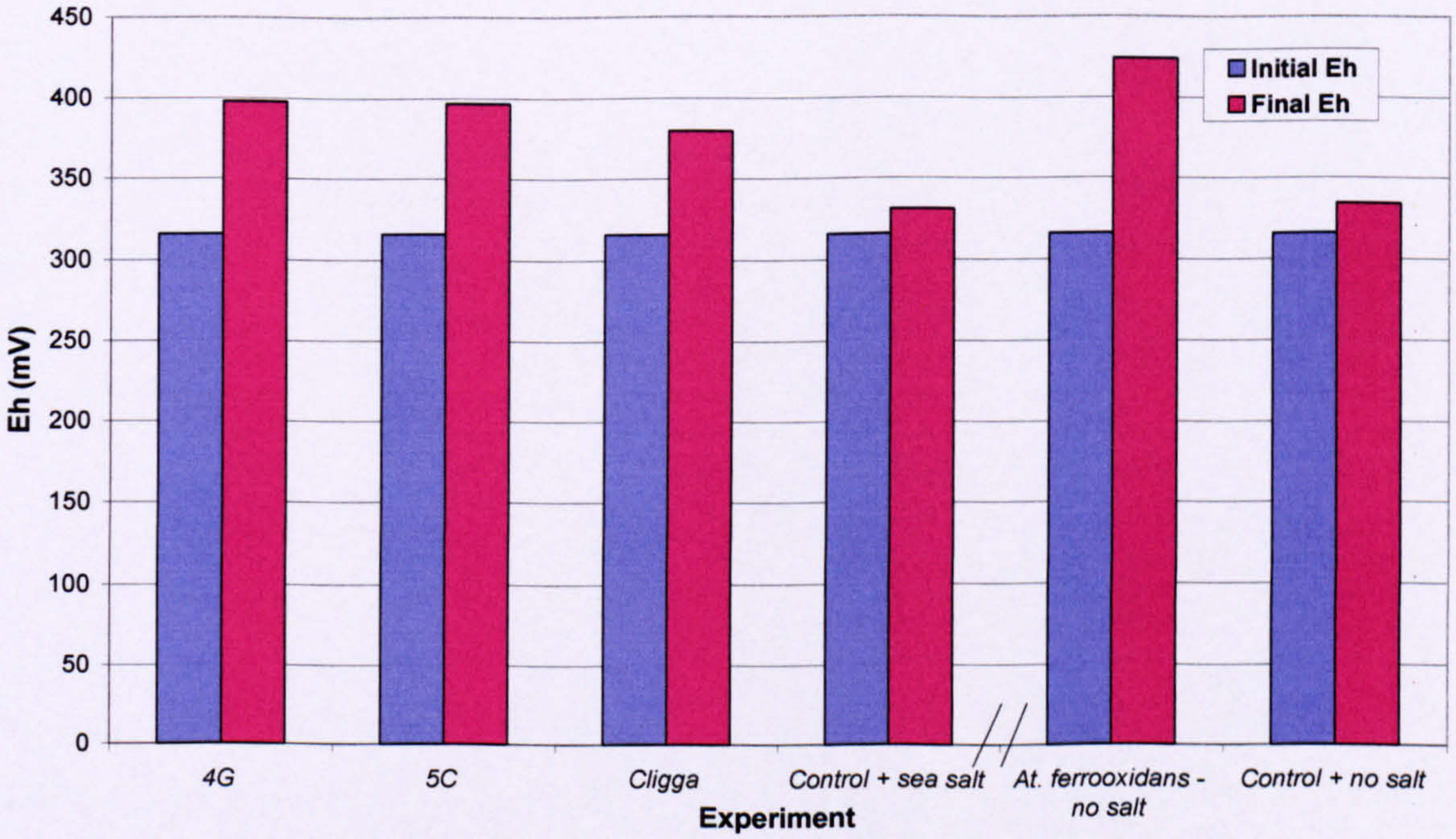


Figure 5.29 Initial and final redox potentials of Somincor copper ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

5.7 Bioleaching of Escondida ore sample by the isolated bacteria

5.7.1 Introduction

The Escondida operation is an open pit copper mine situated 3000 metres above sea level in the Andes mountain range of Northern Chile. The plant and mine are capable of an annual production of more that 800,00 tonnes of copper, in concentrate form. This ore has a copper load of 1.91% and only 2.23% of the ore sample is composed of iron. This mine is close to salt petre deposits that are known to contaminate some of the mine systems in this area (www.chilnet.cl/escondida and Rio Tinto Technology Ltd; Technical Report).

5.7.2 Growth and iron dissolution kinetics of the isolated bacteria when grown on Escondida copper ore

Strains 4G and Cligga exhibited lag periods of three days when grown on Escondida copper ore, and 5C had a lag period of two days (Figure 5.30). All of the strains gave good final cell numbers, but exponential growth rate constants were quite low; 4G 0.60 day^{-1}, 5C 0.65 day^{-1} and Cligga 0.74 day^{-1}. Mean generation times were therefore quite high, being 40, 36.96 and 32.4 hours respectively. Cell number decreased sharply around days eight to ten, after the log phases of the cultures.

Ferrous iron concentration in the cultures of all the isolated bacteria decreased steadily from the beginning of the experiment until around day twenty when it remained relatively unchanged until the end of the experiment (Figure 5.31). Iron dissolution from the ore sample began around day four for all of the strains and increased steadily until day thirty when the total iron in solution was as follows; 4G 0.24 gl^{-1}, 5C 0.27 g l^{-1} and Cligga 0.23 gl^{-1} (Figure 5.32). The iron dissolution of Escondida ore by *At. ferrooxidans* after 30 days was higher than the three isolates with 0.34 gl^{-1} iron in solution, however this was in the absence of sea salts. The overall percentages of iron extracted from the total iron in the ore sample were relatively high as can be seen on Figure 5.33, with extraction percentages of 52.63 % by 4G, 60 % by 5C, 49.75 % by

Cligga and 75.5 % by the benchmark microorganism *At. ferrooxidans* (in the absence of sea salts).

Figure 5.34 shows the change in pH after 30 days of growth of the test bacteria on the Escondida ore sample. The drop in pH observed in all of the test cultures indicates growth of these bacteria and production of H_2SO_4. The redox potential in all of the test cultures increased after 30 days of growth on Escondida ore (Figure 5.35). This indicates the leaching of the ore by the bacteria and the increase of the ferric/ferrous iron ratio in these cultures.

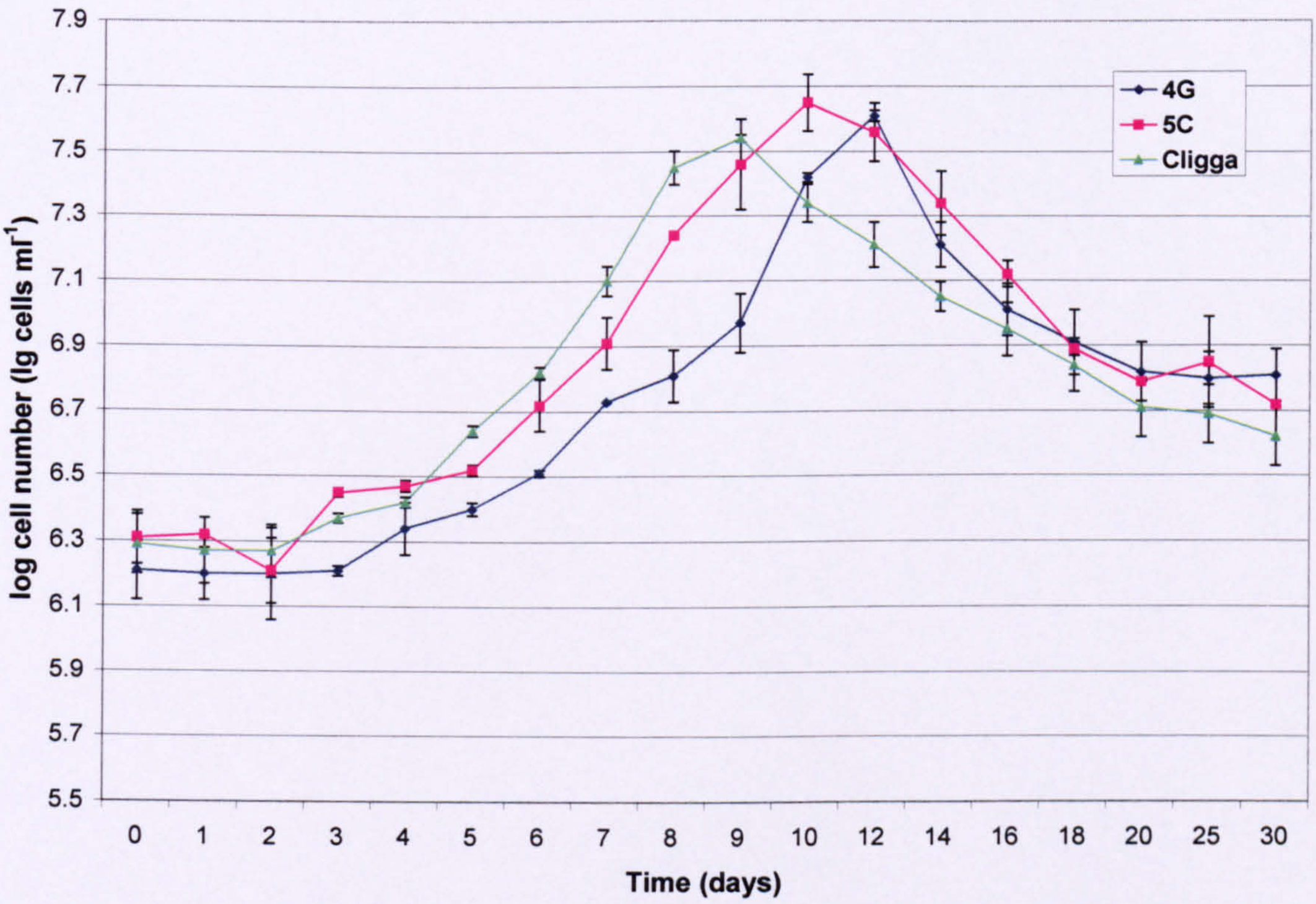


Figure 5.30 Growth of isolated bacteria on 2 % (w/v) Escondida copper ore in medium with 30 gl^{-1} sea salts. Each datum point represents the mean ± standard deviation of duplicate cultures.

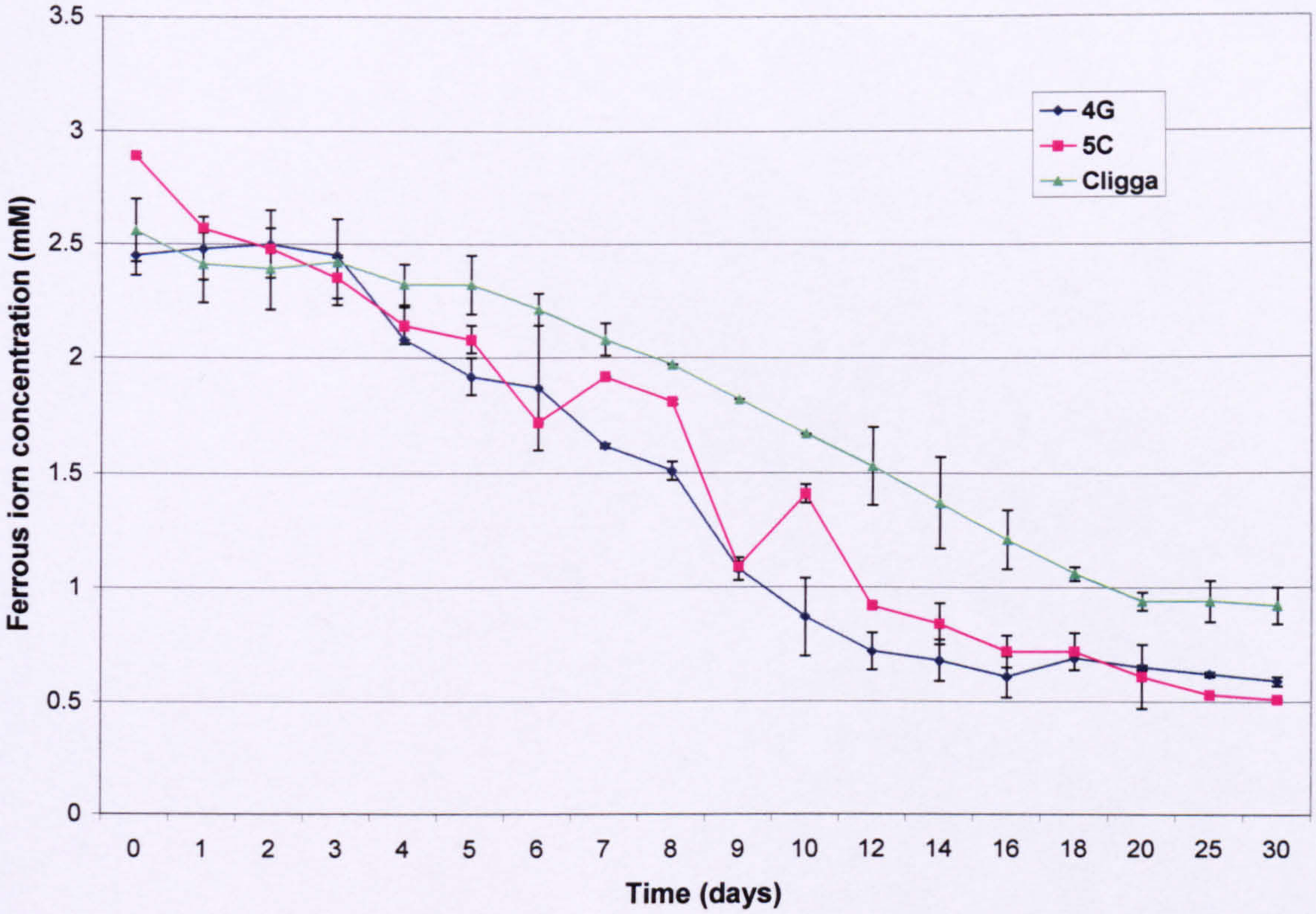


Figure 5.31 Change in ferrous iron concentration in cultures of isolated bacteria grown on Escondida copper ore. Each datum point represents the mean ± standard deviation of duplicate cultures.

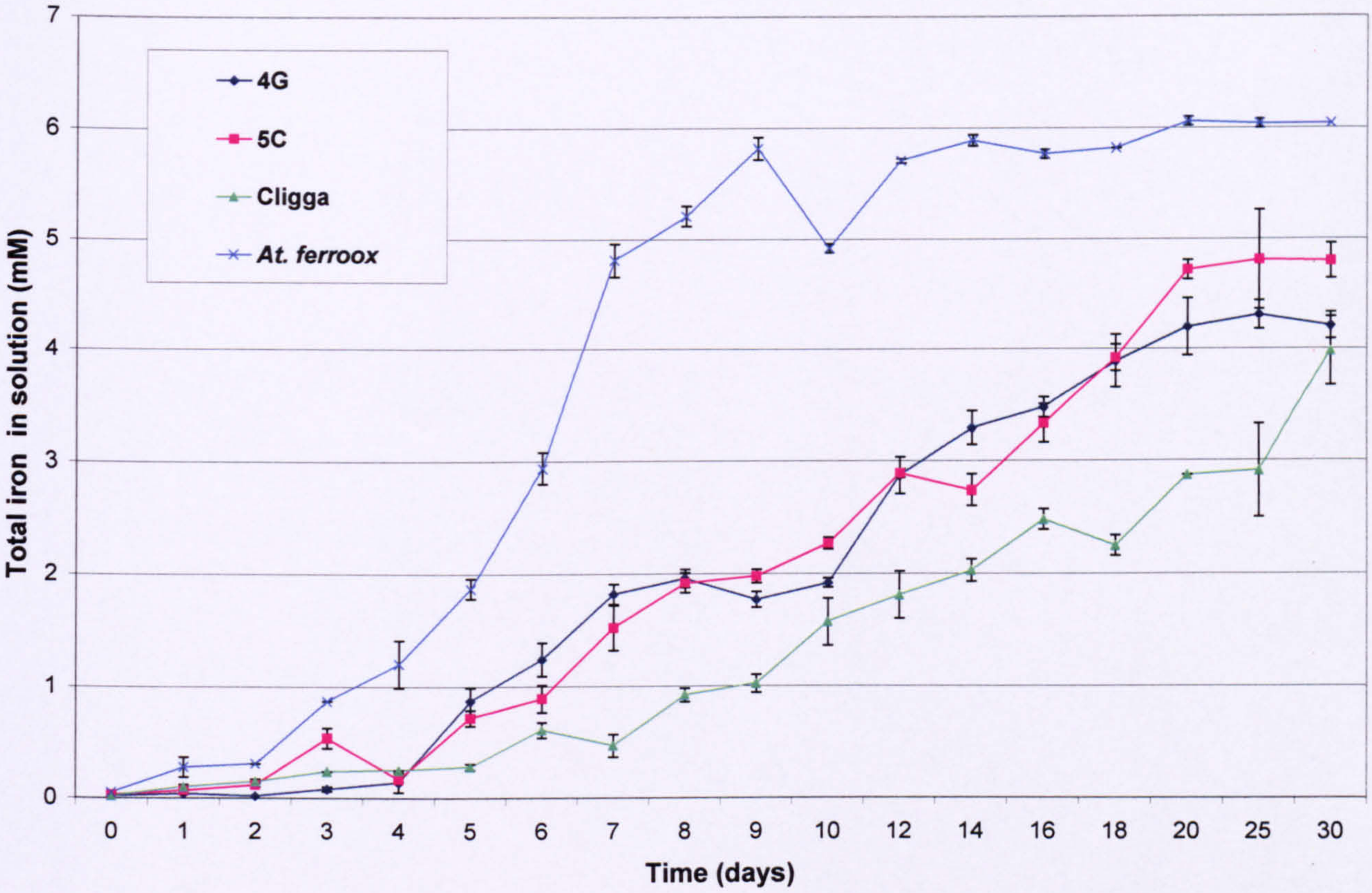


Figure 5.32 Total iron dissolution from Escondida copper ore by isolated bacteria with 30 gl^{-1} sea salts and *At. ferrooxidans* with no added salt. Each datum point represents the mean ± standard deviation of duplicate cultures.

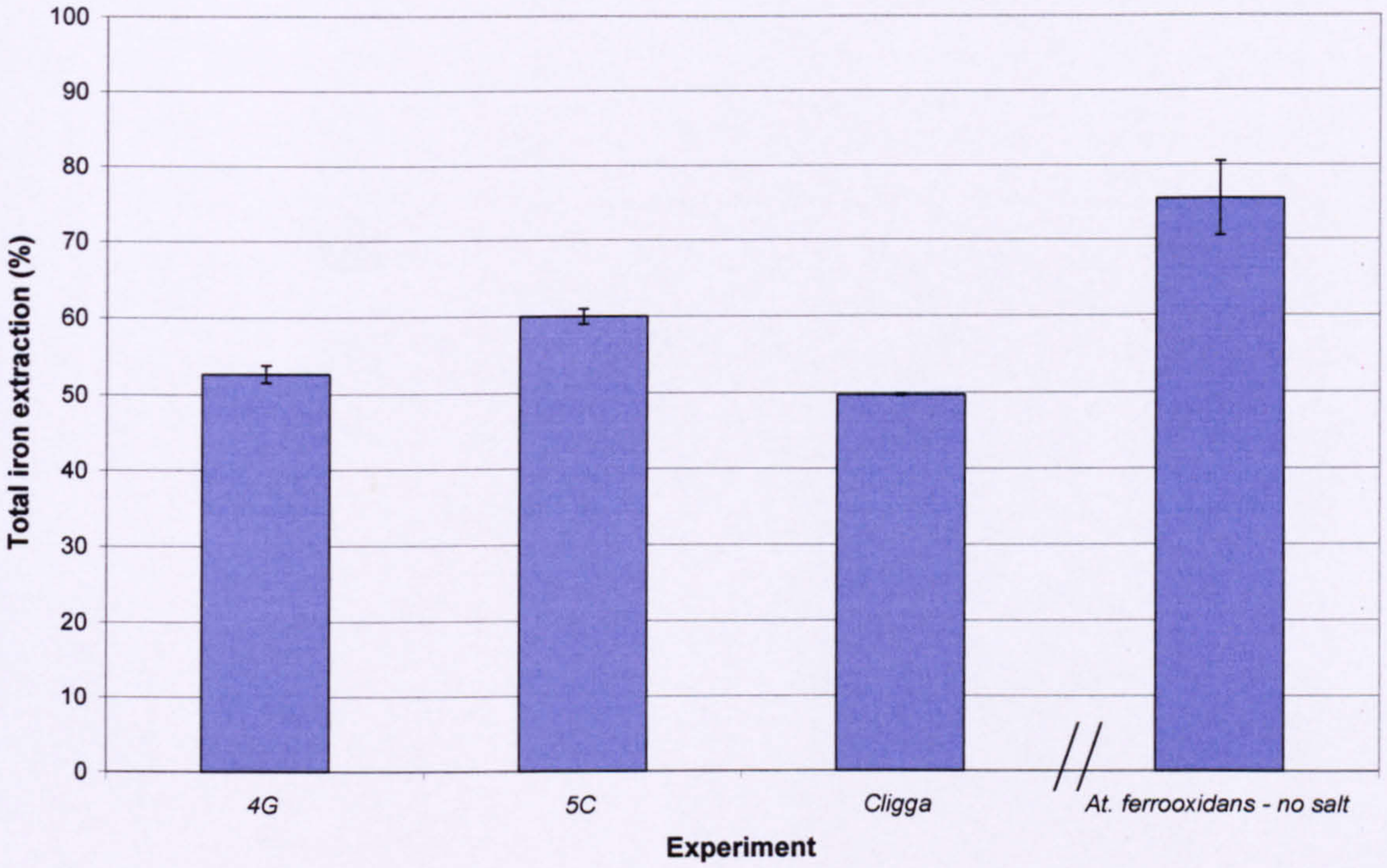


Figure 5.33 Total iron extracted from Escondida copper ore by isolated bacteria and *At. ferrooxidans* as a percentage of total iron in ore sample, after 30 days. Each datum point represents the mean ± standard deviation of duplicate cultures.

Table 5.5 Average and fastest rates of iron dissolution from Escondida copper ore by isolated bacteria *and At. ferrooxidans*

Experiment	Average rate of iron dissolution (mM day^{-1})	Fastest rate of iron dissolution (mM day^{-1})
4G	0.14	0.56
5C	0.16	0.4
Cligga	0.13	0.28
At. ferrooxidans	0.2	1.2

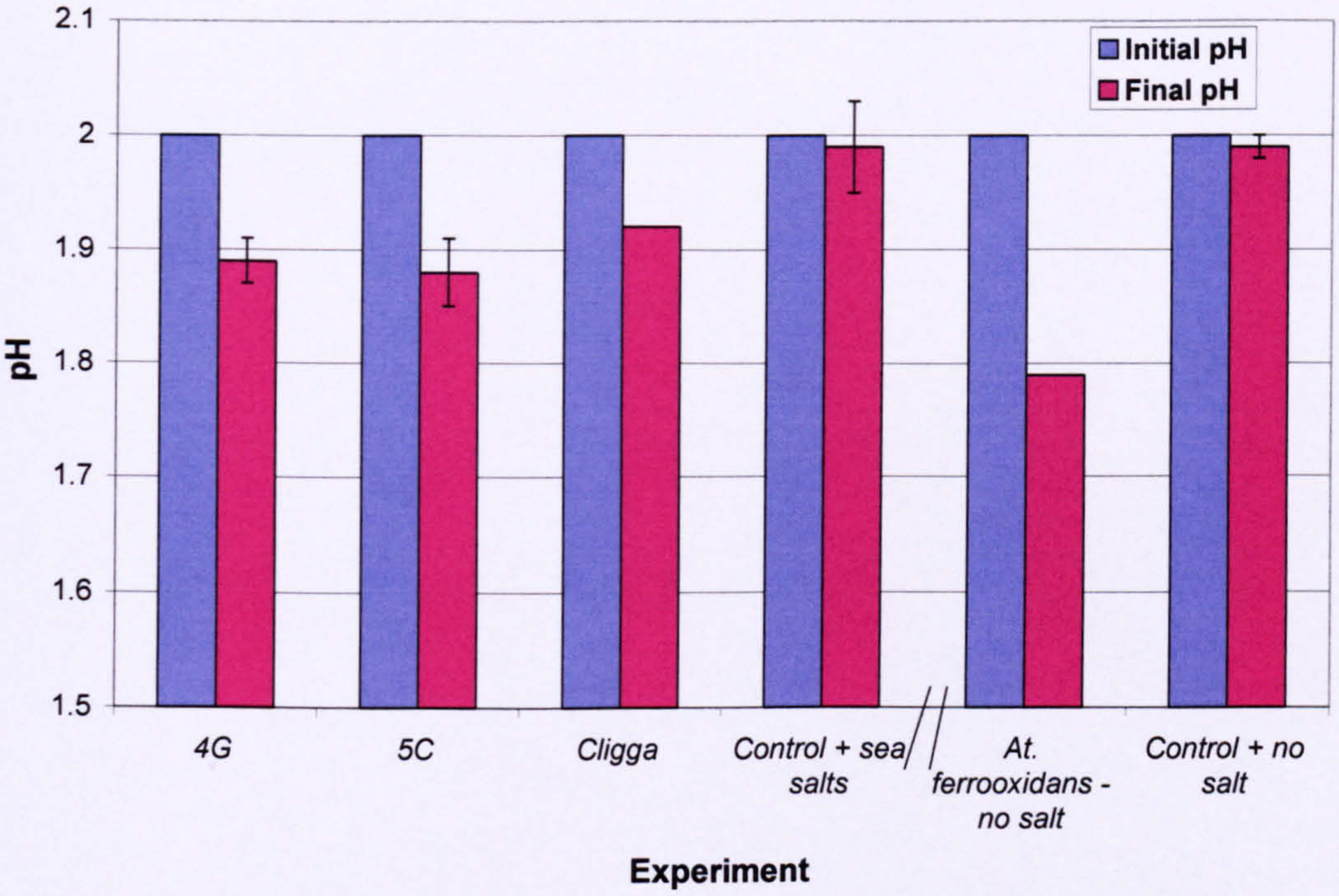
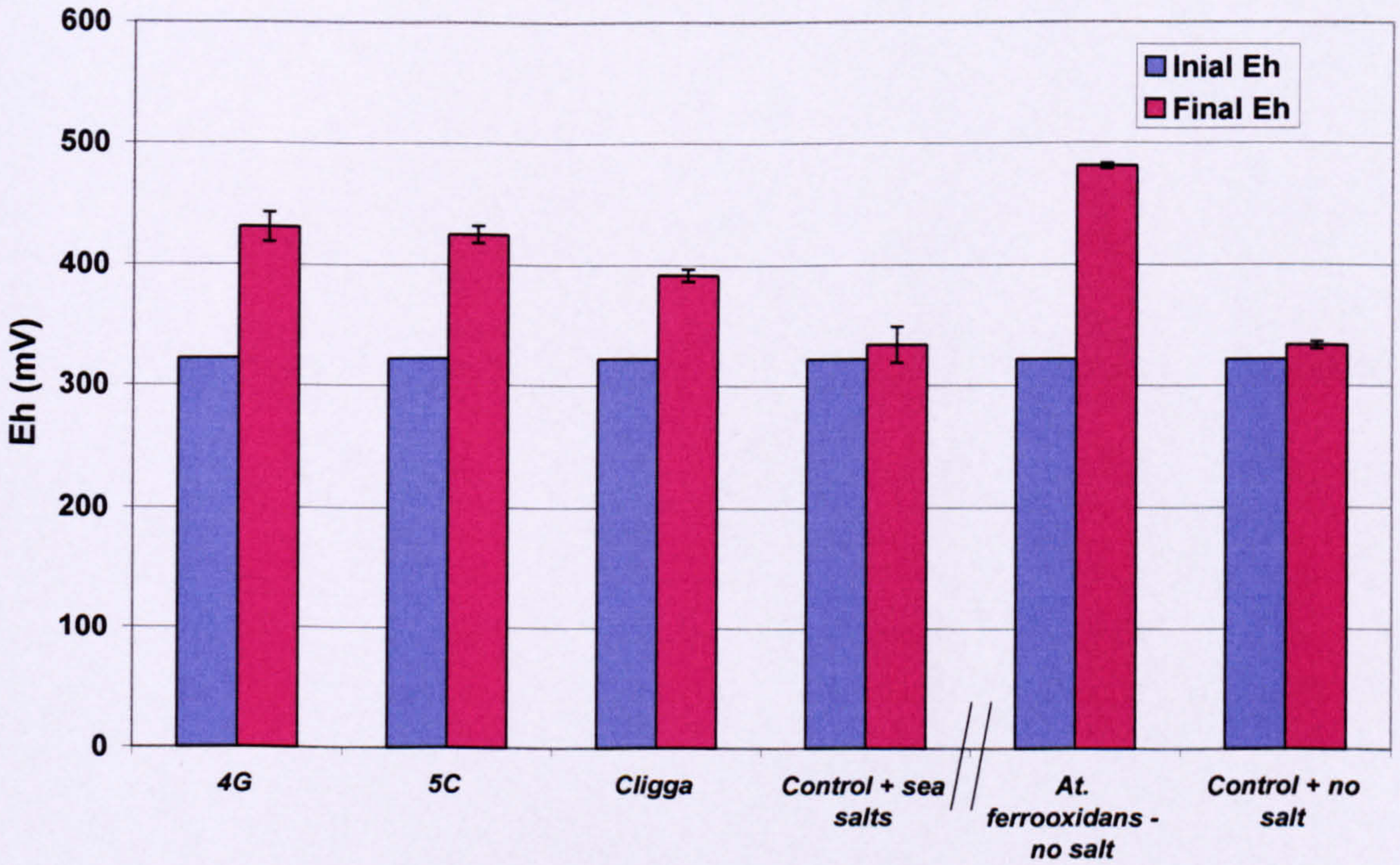


Figure 5.34 Initial and final pH of Escondida copper ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.



5.35 Initial and final redox potentials of Escondida copper ore experiments. Each final datum point represents the mean ± standard deviation of duplicate cultures.

5.8 Comparison of iron dissolution from different ore samples mediated by the test bacteria.

The total amount of iron solubilised by the test bacteria varied between the ore type being leached. It can be seen from Figure 5.36 that the best total iron dissolution occurred from the Lihir ore sample, followed by the Escondida sample. However very poor total iron dissolution was mediated by the test bacteria and *At. ferrooxidans* from the Freeport Final concentrate.

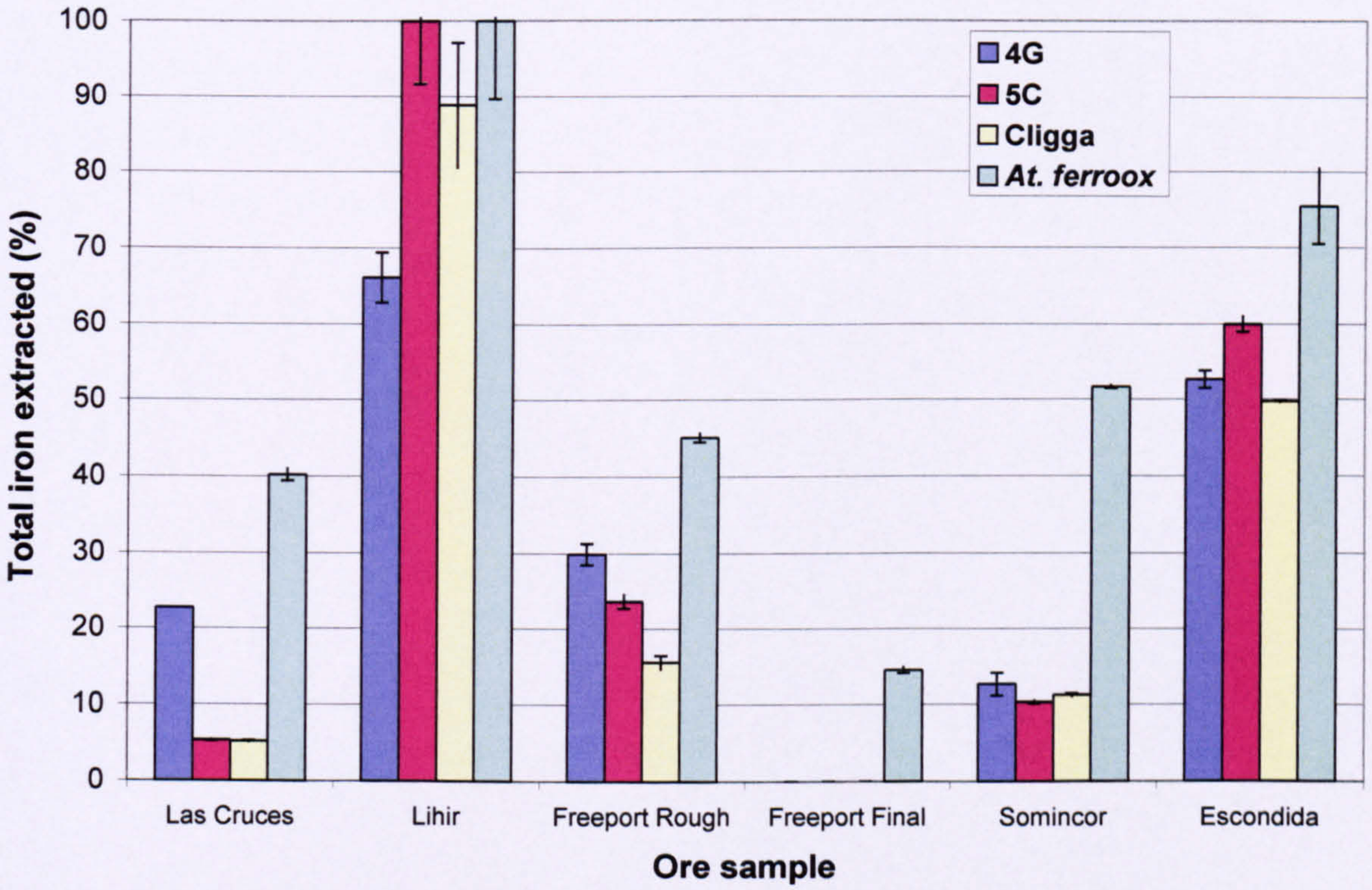


Figure 5.36 Percentage of total iron solubilised from ore samples by isolated bacteria after 30 days growth at 30 g l^{-1} sea salts and 2 % (w/v) ore load. Each datum point represents the mean ± standard deviation of duplicate cultures.

5.9 Discussion

All three of the isolated bacteria were able to grow on all six of the complex polymetallic test ores at an ore load of 2% w/v, with 30 gl^{-1} sea salts in the medium and at an incubation temperature of 37 °C. The strains exhibited the highest exponential growth rate constants on Las Cruces copper ore and Lihir gold ore samples, with the highest rate of growth being exhibited by strain 5C on Lihir gold ore, with an exponential growth rate constant of 1.59 day^{-1} and a mean generation time of only 15.12 hours. Huber & Stetter (1989) report growth kinetics data for a halotolerant iron-oxidising bacterium (*T. prosperus*) grown on mineral ore. The authors reported that *T. prosperus* had a mean generation time of approximately 20 hours when grown on a chalcopyrite, pyrite and sphalerite ore mixture. This generation time is equivalent to an exponential growth rate constant of 1.21 day^{-1}. Therefore, the strains described in this study show higher growth rate constants on certain ore samples than *T. prosperus* at the same salinity and temperature. There are very few growth rate data in the literature on the studies of halotolerant iron-oxidising bacteria grown on mineral ores, and most of the reports show only iron-oxidation kinetics. This therefore means that there are very few comparable data to the growth data contained in this chapter.

Growth on the other ore samples was quite slow with generation times ranging from 32.4 hours (Cligga grown on Escondida ore) to 100 hours (Cligga grown on Somincor ore). However, growth rate constant is not necessarily a measure of the oxidative capacity of a culture and therefore ferrous iron oxidation and dissolution of iron kinetics are of utility when assessing bioleaching potential.

Iron extraction rates varied widely between both test bacteria and ore substrate tested. The bacteria produced the highest iron extraction rates when grown on Lihir gold ore. Strain 5C extracted 100% of the total iron in the culture into solution at a salinity of 30 gl^{-1} and this was equal to the extraction by *At. ferrooxidans* in non-saline medium. Cligga mediated 88.86% extraction of the total iron and 4G mediated 66.10% extraction of the total iron available.

Lihir ore is a low-grade gold ore with 7.76% iron and is highly recalcitrant to normal processing methods and therefore it would be very useful if a high proportion of the iron in this ore could be extracted via pre-treatment with the bacteria isolated in this study. This would therefore leave the gold exposed and highly susceptible to chemical extraction methods, thereby potentially providing a more economic process. In addition, it would be possible to use seawater in the process and with potential to recycle leachate water.

High final iron extraction levels were observed when the isolated bacteria were grown on Escondida ore. Isolated bacterial strain 4G extracted 52.63% of the total iron in the culture, 5C extracted 60 % and Cligga extracted 49.75%. This was compared to 75.5% extraction by *At. ferrooxidans*, again in non-saline conditions.

Deveci (2002) described the effect of salinity on the oxidative activity of acidophilic bacteria. The author described iron extraction rates by a mixed enrichment culture (WJM) from the Wheal Jane mine, Cornwall, with 3% added chloride (NaCl). The total extraction rate at this salinity was 30% of the total iron in a complex Zn/Pb sulphide ore, which had a total iron content of 7.95%. The author reported that the addition of chloride exerted an adverse effect on the oxidative activity of the bacterial cultures, and that long lag periods in the dissolution of iron were observed.

Holden *et al.* (1999) reported the isolation of halotolerant, iron-oxidising bacteria from harbour sediments. However, when the authors carried out bioleaching studies using these bacteria at high salinity (4%) and 2% w/v sediment load, they observed low iron dissolution rates, with only a maximum of 8.15% of the total iron extracted after 20 days.

The differences in iron solubilisation rates observed between the different ore samples may be due to the different mineralogies and chemistries of the samples. High levels of toxic metals present in an ore sample may inhibit the growth of bioleaching bacteria thereby affecting the iron dissolution rates. For example, the Freeport Final concentrate and Somincor ores had high levels of zinc, 2.878 mgg^{-1} and 20.00 mgg^{-1} respectively, as compared to the Lihir ore that had a zinc concentration of only 0.254 mgg^{-1}.

Bioleaching bacteria are able to maintain high redox potentials in leaching systems due to the regeneration of ferric iron (Pronk & Johnson, 1992). The bacterial oxidation of ferrous iron has been found to be a function of the ferric/ferrous ratio, and redox potential falls as the ferric concentration decreases and as a result so too does the overall rate of leaching (May et al, 1997). Redox potential measurements can therefore be used as an indication of the bioleaching conditions within a leach system and can actually determine the degree of leaching of the mineral (Breed & Hansford, 1999).

During the bioleaching experiments outlined in this chapter, redox potential measurements were taken at the beginning and end of each experiment. It was found that a high rate of iron dissolution from ore samples was accompanied by a large increase in redox potential during the leaching experiments. This suggests that ferric iron was being regenerated by the bacteria and was facilitating the chemical leaching of the ore substrates, which concurs with the in-direct mechanism of bioleaching. Redox gradients can greatly influence the growth of iron and sulphur oxidising bacteria in nature and so it is preferable to use bacteria in bioleaching processes that can withstand fluctations in redox potential. The redox potential produced during mineral leaching using *Sulfobacillus* sp. has been found to be lower than that produced by *At. ferrooxidans* or other Gram negative bacteria (Yahya & Johnson, 2002) and this may explain why growth and dissolution rates (and therefore final redox potential) on some metaliferrous ores are lower using the isolated bacteria which are related to *Sulfobacillus* and *Alicyclobacillus* sp than when using *At. ferrooxidans*.

Optimum conditions for growth of the isolated bacteria (including pH, temperature and salinity) were reported in Chapter 3 and these were used during the bioleaching experiments. However, during this study bioleaching parameters were not fully optimised and therefore the observed leaching rates are not necessarily the maximum rates possible. As a result, any future work on the bioleaching potential of these bacteria should first focus on the optimisation of these parameters, thereby potentially improving bioleaching rates.

Chapter Six

Discussion

Chapter Six: Discussion

The isolation and characterisation of novel salt-tolerant, iron-oxidising bacteria has not been widely described in the literature. This is due to a number of different factors, which make the study of this type of bacteria a difficult undertaking despite the interest in these unique bacteria. These factors include the difficulty of culture, relatively long mean generation times and the complex mode of metabolism of these bacteria. Despite the formidable challenge of their study, interest in the occurrence of such bacteria is increasing due to their potential use in biotechnology and their role in the biocorrosion of metal containing structures in marine areas.

Reports suggest these bacteria are ubitiquous in high salt environments and that they play a large part in iron cycling in such environs (Eberhard *et al*, 1995: Gugliandolo & Maugeri, 1993, Hermie, 1997 and Huber & Stetter, 1989). These high salt habitats include marine and estuarine areas, salt lakes and marshes where there is a readily available source of reduced iron and sulphur compounds.

6.1 Isolation sites

Sampling locations were chosen that were known to be areas with high iron and salt concentrations. Since Cornwall, UK has been the site of intense mining activity and many of these mines occurred near or on the coast it was decided to chose sample sites from this area (Pirrie *et al*, 1999 & 2000a & b). Some estuarine sites were also chosen because they were known to have a high level of mine-tailings contamination having been carried down stream from mines by rivers or via industrial accidents (D. Pirrie, Camborne School of Mines, UK, personal communication).

Two of the strains isolated in this study (4G and 5C) were isolated from an estuary (Restronguet Creek) which was highly contaminated with pyritic mine-tailings and acid mine drainage. This contamination occurred in 1992 when acidic metal laden waters overflowed from The Wheal Jane Tin mine located upstream on the Carnon river. The samples from which these bacteria were isolated had red coloured iron oxide veins throughout which indicated the presence of iron tailings and were exposed to tidal seawater. These conditions proved to be a suitable habitat of bacteria that were highly

adapted to growth on iron minerals and in the presence of seawater. These may have been bacteria that originated from the iron-rich mineral environment of the mine upstream and having been introduced into the estuary became adapted to growth at high salinity. There have been reports of the isolation of bacteria of the same genus as 4G (*Sulfobacillus* spp.) having been isolated from the Wheal Jane mine site, although the salt tolerance of these has not been examined (Yahya & Johnson 2002 and Deveci, 2002). Alternatively, the isolated bacteria may have been indigenous to this site and may have been selected under the high metal conditions after acidification by the acid mine drainage contamination.

The strain Cligga was isolated from rock pool scrapings in a disused mine adit, situated in caves that were in tidal contact with seawater. The cave walls were composed of pyritic minerals with various metals including tin, arsenic, silver and copper. The salinity of the seawater from the pool was 4.2% which is much higher than the salinity of the open seawater at this site which was 3.5%. The evaporation of water during the day and subsequent concentration of the dissolved salts may have caused the higher salinity in the pool. There were specks of iron oxide in the pool and this could have been either the product of abiotic iron oxidation or have been mediated by the bacteria present in this locale. Nevertheless, this showed that there was a potential source of iron for energy acquisition by iron-oxidising bacteria.

6.2 Isolation media

It is reported that 99.9% of all the bacterial species on the earth are yet to be discovered (Jannasch, 1984) and isolated and this is due, in no small part, to the use of isolation and enrichment culture methods that are not able to support the growth of certain bacteria. However, improved molecular methods have indicated the presence of certain types of bacteria which have not yet been cultured and different culture methods are constantly being developed to try to isolate these elusive microorganisms (Amann *et al*, 1992, Harrison, 1984, Stackebrandt & Rainey, 1995).

Solid medium is the traditional isolation medium for most bacteria due to the fact that single colony formation facilitates obtaining pure cultures from mixed environmental enrichments. However, acidophilic bacteria have proved difficult to grow using these traditional methods, and although novel solid media have been developed for this purpose and present an improvement in culture methods (Johnson, 1995), these are not conducive to the isolation of halotolerant bacteria.

During this study no bacteria were isolated from the sample on overlay plates and seawater medium solidified with agarose (TSM plates). This lack of growth may have been due to nutrients being complexed within the agarose matrix and therefore being less available for bacterial growth. For example, Meargy (1995) noted that metal sensitivity levels measured in solid media might not be accurate due to the metals forming complexes with the medium. It was also found in the present study that the *Acidophilum* sp. in the bottom layer of the overlay method was sensitive to elevated salt levels and so this method was not of use.

Successful enrichments were obtained using pyrite saline medium at pH 2.0 with 30 gl^{-1} sea salts. These enrichments were incubated statically at 28°C. Repeated sub-culture produced pure cultures and the purity of these was checked by single colony formation on solid medium that was developed during this study. The use of successive dilutions in this way should yield an isolate that was a numerically dominant member of the initial enrichment culture and therefore has been found to be a successful method of bacterial isolation (Plumb *et al*, 2002). Of all the enrichment media types used, the pyrite cultures were the most successful, possibly due to the similarity between the

chemical composition of these and the sample environments (e.g. pyritic minerals and presence of seawater).

It was decided to use sea salts in the enrichment medium instead of sodium chloride because the sample sites were in contact with seawater and niche mimic culture methods have been proved to be more successful than other methods of isolation. Most of the reports of isolation attempts of halotolerant acidophiles used NaCl in enrichment media. This may explain why many of these attempts have produced bacteria that have salt concentration optima for growth that are well below that of their original environment (Holden *et al*, 1999; Huber & Stetter, 1989; Kamimura *et al*, 2001 and Smith & Finazzo, 1981). For example, Smith and Finazzo (1981) isolated *T. intermedius* that has a NaCl optimum for growth of 1% but was isolated from salt marsh sediment with an interstitial salinity of 3%.

It has been reported that KCl plays an important role in the ability of bacteria to grow at elevated salinity (Kushner, 1988; Madigan & Oren, 1999; Kogure, 1998 and Suzuki *et al*, 1999) and may help bacteria maintain their internal solute potential. The monovalent cation K^+ protects against cell lysis by increasing intracellular osmotic pressure and stabilises the cell membranes of bacteria (Smith & Finazzo, 1981). Seawater has a high concentration of KCl (Brown *et al*, 2002) and the availability of this compound in the enrichment and growth medium of the isolated strains may confer a higher tolerance to elevated sodium or chloride toxicity.

6.3 Growth physiology

The growth of the three isolated strains was tested in medium either with no added salt source or the addition of 30 gl^{-1} NaCl or sea salts and it was found that sea salts was the optimum salt source. The growth rate in sea salts medium of 4G was 2.5 times, 5C 4.1 times and Cligga 3.8 times that of culture in NaCl medium. However, some growth was observed in medium without an added salt source (albeit at a greatly reduced rate) and therefore these strains cannot be termed halophilic or marine as they do not have an obligate salt requirement for growth. This is in accordance with the definition set out by Macleod (1965) describing the salt requirement of a marine bacterial species.

However, when growth at different salinity values was assessed, it was found that these isolates had optimal growth at 30 gl^{-1} sea salts and growth rate decreased with decreasing salt concentration. This was the first report of the isolation of halotolerant, iron-oxidising acidophilic bacteria whose optimum growth rates were observed at salt concentrations that were equivalent to those of the environments from which they were isolated.

The majority of the other halotolerant acidophiles, which have been described in the literature, have salinity optima that are less than their natural environments. These environments are usually characterised by having available solid-strata, for example sediment material or rocks and this may induce the formation of exopolysaccharides by growing acidophiles in contact with such surfaces. The biofilm thus formed may slow down the diffusion of sodium and chloride ions and reduce the exposure of the cells to toxic concentrations of these ions. Therefore, whenever the growth of these bacteria at different salt concentrations are assessed in liquid medium without solid-strata, biofilms are not formed and growth at high salinity may be slower without the protective effect of the biofilm components. Similarly, Cameron *et al* (1984) reported that clay minerals had a protective effect against salt toxicity during halotolerance studies on isolated acidophiles.

The isolated bacterial strains grew under heterotrophic conditions using yeast extract as the sole energy source, and under autotrophic conditions using pyrite as a energy source (e.g. the enrichment medium) and also grew under mixotrophic growth conditions, using a mixture of these substrates. Long lag periods were observed in heterotrophic

cultures as compared to mixotrophic cultures but overall exponential growth rates were higher in heterotrophic growth medium. These long lag periods may be due to the time needed for the switching on of genes involved in heterotrophic growth. However, heterotrophic growth generates more energy than an inorganic source such as pyrite and so the higher growth rate when grown on yeast extract is not wholly unexpected. The ability to grow mixotrophically is indicative of *Sulfobacillus*-like bacterial species. *Sulfobacillus* sp. have been reported to perform inefficient carbon dioxide fixation and display increased growth rates in the presence of an organic source of carbon such as yeast extract (Yahya & Johnson 2002).

Many *Sulfobacillus* sp. and *Alicyclobacillus* sp. are moderately thermophilic, e.g. *S. thermosulfidooxidans*, *S. acidophilus*, *A. cycloheptanicus* and *A. hesperidum* (Goto *et al*, 2002; Dufresne *et al*, 1996). However, strains 4G, 5C and Cligga grew optimally at 37°C and were enriched at 28°C. There have been two reports of mesophilic *Sulfobacillus* sp. (*S. montserratensis* and *S. ambivalens*) and one of an *Alicyclobacillus*-like bacterium (*S. disulfidooxidans*). The former two mesophiles were isolated from areas of high temperature on the island of Montseratt (55°C) and the authors noted that it was unexpected to isolate mesophiles from this site. However, this shows that the temperature of the indigenous environment is not always the optimum temperature for growth.

The temperatures of the environments from which these three strains were isolated ranged from 2°C to 5°C and therefore are growth temperatures indicative of psychrophilic growth. However, the oxidation of iron and sulphur compounds generate a great deal of heat and these bacteria may have been growing in microenvironments where the local temperature was increased by this metabolic reaction, thereby facilitating optimal growth. Temperatures within bioleach heaps, dumps and coal spoilage heaps are usually around 35°C to 45°C but can reach up to 50°C, via the generation of heat from microbial metabolic processes (Bustos *et al*, 1999; Norris *et al*, 1986; Rawlings, 2002 and Rawlings & Silver, 1995).

Since these bacteria may be of potential in biomining processes, growth was assessed at 28°C, 37°C, 45°C and 50°C. Limited growth was observed at 40°C and no growth was observed at 50°C with an optimum temperature for growth of 37°C. As these isolated bacteria are spore forming bacteria they may have been present as spores in the sample

material as opposed to viable cells or may have been dormant cells or growing very slowly at the low temperature of these sites.

The solubility of oxygen in seawater decreases with increasing salinity and increasing temperature (Table 6.1), therefore the oxygen availability to cultures of the isolated strains may be a growth rate-limiting component of the cultures. This is also true for the solubility of carbon dioxide which these bacteria need to fix for incorporation as cellular carbon. Higher growth rates may have been observed if the cultures were sparged with air or carbon dioxide. During bioreactor studies using *T. prosperus* grown at 30 gl^{-1} sea salts, sparging with air was found to increase the growth rate and final biomass obtained (this study, results not shown).

Table 6.1 The solubility of oxygen in seawater as a function of temperature and salinity (Dexter, 1995).

Temperature (°C)	Solubility of O_2 (mll^{-1}) at different salinity		
	0% salinity	2.4% salinity	3.6% salinity
0	10.2	8.7	8
15	7.1	6.1	5.7
30	5.3	4.6	4.3

The strains had pH optima of around pH 2.0 and grew very slowly at pH 1.0. Seawater is a very efficient natural buffer and has an average pH of approximately 8.0 depending on the site (Brown *et al*, 2002) but acidophiles do not grow optimally at this pH as they grow optimally at pH values under pH 3.0 (Johnson, 2001).

However, the formation of localised microenvironments may provide a low enough pH to allow the growth of these bacteria via the production of H_2SO_4 by the metabolic reactions of the bacteria and protection of these sites. Protective microcosms may be established by biofilm formation, by the excretion of exopolysaccharides around the bacteria (as in the direct mechanism implicated in bioleaching – see Section 1.4.1) and the acid may be concentrated in these locales, providing favourable conditions for growth.

Hydrothermal vent systems and fumaroles may have acidic environments due to the production of H_2S and other sulphur compounds being converted into H_2SO_4 on reaction with seawater. These therefore may provide conditions for the growth of acidophiles (Eberhard *et al*, 1995; Gugliandolo & Maugeri, 1993; Harmsen, *et al*, 1997; Prieur, 1997). The pH value decreases during bioleaching processes and therefore bacteria must tolerate these low pH values if they are to be of use in biomining processes. Since the isolated strains tolerated low pH, this is one parameter that may makes them suitable for use in biomining processes.

Table 6.2 shows the growth rate constants and mean generation times of the isolated salt-tolerant bacteria in different media and under different conditions. This table clearly shows that optimum growth conditions of those tested (including growth on the metalliferrous ores) was the heterotrophic medium with yeast extract as an energy source with 30 gl^{-1} sea salts, at pH 2.0 and a temperature of 37°C.

Table 6.2 Mean growth rate constants (k) and mean generation times (td) of the isolated salt-tolerant bacteria when grown in different media with different conditions

Medium	4G		5C		Cligga	
	k (d^{-1})	td (h)	k (d^{-1})	td (h)	k (d^{-1})	td (h)
YSM (heterotrophic)	3.41	7.04	3.55	6.76	3.12	7.69
PSM at 37°C (mixotrophic)	1.21	19.77	1.42	16.69	1.25	19.25
PSM at 28°C (mixotrophic)	0.98	24.49	1.15	20.87	1.05	22.86
Las Cruces ore	0.86	27.84	1.35	17.76	1.03	23.28
Lihir gold ore	1.56	15.36	1.59	15.12	1.45	16.56
Freeport Rough feed ore	0.52	46.08	0.40	60.00	0.49	48.98
Freeport Final concentrate ore	0.5	48.00	0.33	72.72	0.32	75.00
Somincor ore	0.38	63.12	0.31	77.52	0.25	100.00
Escondida	0.6	40.00	0.65	36.96	0.74	32.40

As previously mentioned, extremophiles thrive in environments with more than one extreme, which makes their adaptations even more complex. There has been little research into the various interactions of these extremes in such bacteria, and usually only one extreme is investigated during research. As a result of such observations, work has been carried out on the construction of a model that represents these extremes in three dimensions. Julian Wimpenny and his lab members have constructed a three-dimensional model of the accepted limits of bacterial life for pH, temperature and salt concentration (J. Wimpenny, Cardiff University, UK, personal communication, 2002).

This model has been constructed with a view to not only bringing together information on the physico-chemical limits of life on earth but to also use these models to predict where life may exist outside our planet. These researchers used data regarding extremophilic bacteria, found in the International Journal of Systematic Bacteria and the journal Extremophiles. As a result of this search, Wimpenny noted that the previously characterised bacteria clustered around neutral pH and low salinity and that there is very little data representing bacteria that grow at low pH and high salt concentration. The investigators noted that it was likely that such bacteria were widespread but were resistant to current isolation methods. As a result the investigators extrapolated their constructed models to include these environments.

The halotolerant, iron-oxidising bacteria 4G, 5C and Cligga, isolated during this study would fit in the extrapolated area in the model. This highlights that the novel physiology represented by these bacteria is really at the outer reaches of the generally accepted limits of life, and they may offer an idea as to the type of life that may exist in similar environments outside our planet.

Since most isolation methods select for certain bacteria and therefore eliminate the possibility of isolating others, there is a need to assess novel culture methods for the isolation of difficult to culture microorganisms and elucidate their physiological responses as a result of their extreme environments. Advances in molecular methods have shed light on the phylogeny of so called 'unculturable' bacteria; however, these techniques cannot confirm optimum chemical, physical or biological growth conditions. Elucidation of these factors is extremely important in understanding the role these bacteria play in their environments and the potential use of such bacteria in biotechnological processes.

6.4 Phylogenetics of the isolated bacteria

The isolated bacterial strains were similar to *Sulfobacillus*- and *Alicyclobacillus*-like species with regard to their morphology, spore-formation, mode of growth and Gram-stain characteristics. The phylogeny of these bacteria was assessed by the sequencing of the 16S rDNA regions of the genome and comparing these to 16S rDNA from strains characterised in the nucleotide databases. It was found that strain 4G was a novel species within the genus *Sulfobacillus,* and showed 96% 16S sequence identity with *S. sibiricus* (thermophile) and 94% sequence identity with *S. montserattensis* (mesophile). The tentative species name *Sulfobacillus halodurans* is suggested for this strain which refers to the halotolerance of this strain (halo – salt and durans – tolerant). Strain 4G is the first halotolerant *Sulfobacillus* sp. with its 16S rDNA data displayed on Genbank.

Strain 5C is a novel strain within the genus *Alicyclobacillus* and shares 95% 16S rDNA sequence homology with an *Alicyclobacillus* sp., which was isolated from a thermal spring in Alaska. The tentative species name *Alicyclobacillus halodurans* is suggested for strain 5C, again referring to the halotolerance of this bacterium which makes it distinct within the genus *Alicyclobacillus*.

Strain Cligga also shows high sequence identity (97%) with an *Alicyclobacillus* sp. in particular to the strain AGC-2, which was isolated from a thermal spring in Alaska. However, the characteristics and species name has not been reported for strain AGC-2 and as strain Cligga seems to be a member of the same species, a name has not been suggested for this strain. However, more biochemical and other taxonomic data are needed on these three strains before species designations are formally attempted.

6.5 The potential use of the isolated bacteria in biomining processes at elevated salinity

All three of the isolated bacteria were able to grow on all six of the complex polymetallic test ores at an ore load of 2 % (w/v), with 30 gl^{-1} sea salts in the medium and at an incubation temperature of 37 °C. The highest rates of iron dissolution were obtained in cultures grown on Lihir gold ore, which is a highly recalcitrant ore sample. Total iron extraction values from this ore (as a percentage of the total iron in the ore sample) were 66.10% by strain 4G, 100% by strain 5C and 88.86% by strain Cligga. *At. ferrooxidans* mediated 100% iron extraction from Lihir ore. However, this was in medium with no added salt and due to the toxicity of Cl^- to this bacterium, no growth and no iron-oxidation would have been observed at the salt conditions preferred by the three strains. Table 6.2 shows the growth rate constants and mean generation times of the isolated bacteria in different media and under different conditions. This table shows that the growth rate constants of the bacteria in medium with Lihir ore were higher than those observed in the same medium with pyrite or the other ore samples. This suggests that there may be a component present in the Lihir ore that may serve to increase the rate of growth of these bacteria and which was not present in the other ore samples.

Total iron extraction values from Escondida copper ore sample were 52.63% by 4G, 60% by 5C and 49.75% of the total iron was extracted by strain Cligga. *At. ferrooxidans* mediated 75.5% extraction of the total iron available in cultures of the Escondida ore sample, but again this was in medium with no added salt and this bacterium would not have grown at the culture conditions of the three isolated strains.

It is suggested that high levels of certain metals present in some ores may have been toxic to the test bacteria and may therefore have had a detrimental effect on the dissolution of the ore sample. Therefore, the levels of tolerance of the isolated bacteria to heavy metals should be tested to ascertain whether sensitivity to these metals affected the iron solubilisation rates.

However, extraction rates may be increased by optimisation of the conditions of bioleaching/biooxidation. Table 6.3 outlines the conditions that affect bioleaching efficiency. Further investigation of these factors may further improve the leaching rates obtainable using the test bacteria.

There are very few comparable data available in the literature, regarding the bioleaching kinetics of halotolerant bacteria grown on metaliferrous ores and those that have been reported have used different conditions to those used in this study (Deveci, 2002; Holden *et al*, 1999 and Huber & Stetter, 1989). However, these reports can be used as a relative comparison.

Deveci (2002) described 30% extraction of the total iron available in a Zn/Pb complex ore sample (with a total iron content of 6.95%) at a NaCl concentration of 3%, while Holden *et al* (1999) reported that only 8.15 % of the total iron in samples of harbour sediments was extracted after 20 days of bioleaching at 4% salinity and 2% ore load (w/v). These reports show a lower level of iron-oxidation rates than was observed with the halotolerant strains characterised in this study. However, further analysis of the levels of solubilised target metals may shed further light on the utility of these bacteria in these processes. However, as iron extraction is usually proportional to metal solubilisation, these results show the high potential of these isolated bacteria in high salinity bioleaching of the test ore samples.

The halotolerant, iron-oxidising, acidophilic bacteria described in this study, were all isolated from environments with low pH, high metal contents and high redox potential. Subsistence in environments with these extreme conditions has allowed the refinement of microbial mechanisms involved in the efficient utilisation of metaliferrous minerals for growth. The high adaptation to their environment has provided unique bacterial physiology that can then be harnessed by biotechnology to improve industrial processes. The combination of the ability to grow under these conditions as well as conditions of high salinity, has further broadened the biotechnological potential of these bacteria and may lead to the economic application of these bacteria in the biohydrometallurgical industry.

Table 6.3 Factors that affect bioleaching efficiency. These factors must be further optimised to increase the bioleaching/biooxidation efficiency of the test bacteria.

Factor	Effect
Temperature	Variable solubility of essential gases for bacteria; reactivity of component chemicals affected
Salinity	Different salinity will change the buffering capacity of medium, therefore affecting the pH of leaching solution; salinity affects solubility and therefore availability of oxygen; NaCl, artificial and natural seawater all have different buffering capacities and therefore will produce different metal oxidation kinetics.
pH	Bacteria used in bioleaching experiments all have different optimum pH and all alter the pH during growth to different levels, therefore initial pH can affect the growth and iron oxidation kinetics of the leaching reaction and it can affect the effectiveness of mixed culture growth and select for certain bacteria.
Iron concentration and speciation	Ferric iron effectively catalyses the dissolution of metaliferrous ores (the use of ferric lixivant for abiotic dissolution of pyrite etc); some bioleaching bacteria use ferrous iron as their electron source and therefore need high levels of ferrous iron to grow; some bacteria used in these processes may be inhibited by high levels of ferric ions.
Concentrations of heavy metals present	The solubility of some metals increases with decreasing pH and levels may increase in medium and these potentially toxic levels may inhibit the growth of the bacteria.
Composition, mineralogy of ore substrate	Lowering the particle size of the ore, which thereby creates a greater surface area for microbial attack, can increase the rate of dissolution of metaliferrous ores.
Electrochemistry of leaching conditions	Certain redox chemistry favours leaching processes and these must be optimised to improve microbial biomining processes.
Oxygen and carbon dioxide availability	Availability of O_2 and CO_2 is a rate-limiting factor in the solubilisation of ores by bacteria. Sparging with air can increase growth rates and iron-oxidation rates of bacteria grown on ore samples.
Shaking speed	High shaking speed (above 200 rpm) was found to be detrimental to test bacteria during bioreactor studies (data not shown). These bacteria suffer from shear forces and may be lysed by the edges of the ore particles.
Bacteria	Different bacteria exhibit totally different iron and reduced sulphur oxidation kinetics, due to their differing affinity for certain types of substrate; microbial consortia used must be assessed and optimised to increase bioleaching rates. This might include the assessment of mixed culture consortia, thermophiles or mixotrophs in biomining processes.

6.6 Suggestions for further study

During this study many ideas for further study were brought to light; as Thorstein Veblen (1857-1929) once stated 'the outcome of any serious research is to make two questions grow where only one grew before'. Therefore, presented below are some of these suggestions that were not pursued due to time constraints.

- It has been suggested that tolerance to chloride salts may confer cross-resistance to nitrate salts (David Barr, Rio Tinto Plc. Personal communication, 2001) and that some mine sites such as the Escondida operation in Chile are contaminated with nitrate salts as well as sodium chloride. Therefore, the assessment of the growth of the newly isolated strains at different levels of nitrate salts would provide information of the utility of these strains in such contaminated mine sites.

- Pilot studies showed that increased levels of potassium chloride increased the tolerance of *T. prosperus* and the isolated bacterial strains to sea salts (this study, results not shown). Since KCl has been found to be important in the maintenance of internal cytoplasm water potential, the investigation of the extent of this tolerance at elevated KCl levels would be of utility in elucidation of osmoregulation mechanisms.

- Increased sulphate levels may have an advantageous effect on levels of halotolerance, therefore, the growth of these halotolerant bacterial strains should be assessed with different levels of sulphate in the medium.

- The extent of dissolution of iron from mineral ores was affected by the sporulation of the test bacteria. It has been reported that the addition of yeast extract at different stages of growth may delay sporulation in *Sulfobacillus*-like bacteria (Bogdanova *et al*, 2002). Therefore, it would be of interest to test whether this worked with the isolated bacteria and what effect was produced regarding the rate of iron dissolution from the ores.

- The use of gradient plates for isolation of this type of bacteria should be used to assess the physiological diversity in a given environment. Using these plates provides two-dimensional diffusion gradients for two environmental variables such

as pH and salt concentration (Wimpenny & Waters, 1984). Therefore, the optimum growth parameters of bacteria within the environmental samples, with regard to these two variables, could be estimated and these colonies sub-cultured into the corresponding liquid medium. This would cut down on time needed for enrichment and would provide a quick overview on the diversity of the environment of interest.

6.7 Conclusions

Three strains of halotolerant, iron-oxidising bacteria were isolated and characterised during this study. They were found to be mesophilic, aerobic, rod-shaped Gram-positive bacteria. They all had growth optima of 37°C, pH 2.0 and 30 gl^{-1} sea salts. After phylogenetic analysis, strain 4G was found to be a novel *Sulfobacillus* sp., 5C a novel *Alicyclobacillus* sp. and Cligga was also found to be an example of an *Alicyclobacillus* sp. All three strains grew autotrophically on pyrite, heterotrophically on yeast extract or mixotrophically using combinations of these substrates. They also grew on various metaliferrous mineral ore samples, augmented with yeast extract and solubilised the iron in these ore samples. The halotolerant bacterial strains exhibited high dissolution rates from Lihir gold ore and Escondida copper ore at high salinity and these rates demonstrated the potential of these strains in biomining processes at high salinity.

Due to the growth of the biomining industry, new microorganisms that are capable of leaching metals from sulphidic ores are constantly being sought. Subsequently the diversity of bacterial consortia that may give significant process advantages in biohydrometallurgical technologies is continually increasing. The diversity of the environments from which these microorganisms are being isolated is widening all the time and more advanced microbiology and molecular ecology techniques are enabling more effective culture and identification of these traditionally difficult to culture bacteria.

The isolation of halotolerant, iron-oxidising, acidophilic bacteria has further extended the currently accepted limits of life. Further characterisation of the physiology of the bacterial strains that were isolated and described in this study, and optimisation of their growth conditions, will not only increase their biomining potential, but will shed light on the role of these bacteria in biogeochemical cycling of iron in areas of high salinity.

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Appendices

Appendix A: Locations of sampling sites in Cornwall, UK

A.i: Locations of sampling sites within Cornwall

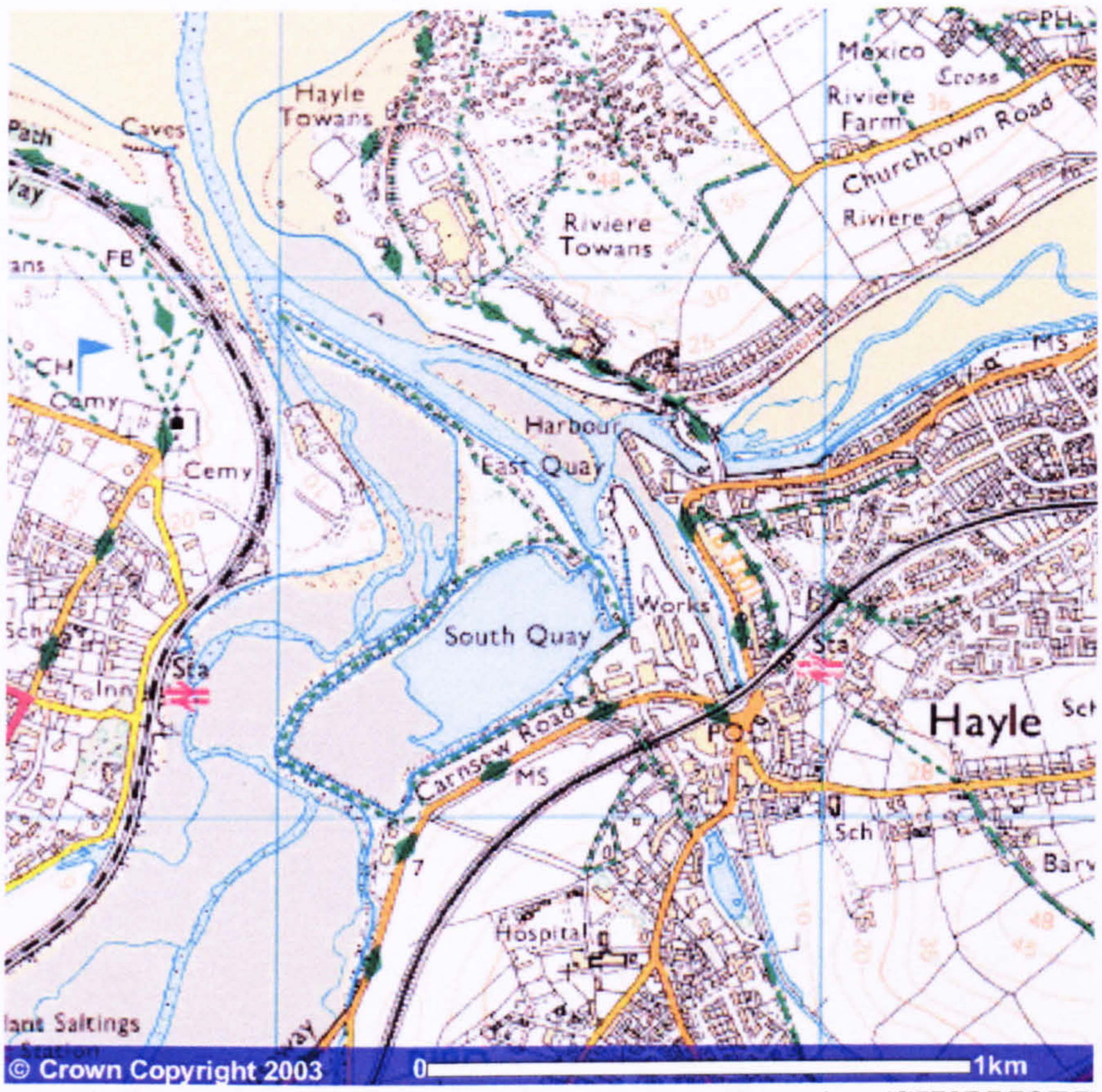
ii-xi **Locations of sample sites within Cornwall UK**

A.ii: Location of Calenick Creek sampling site (SW827432)

A.iii: Location of Cligga Head sampling site (SW737537)

A.iv: Location of Devoran sampling site (SW797387)

A.v. Location of The Gannel Sampling site (SW801609)

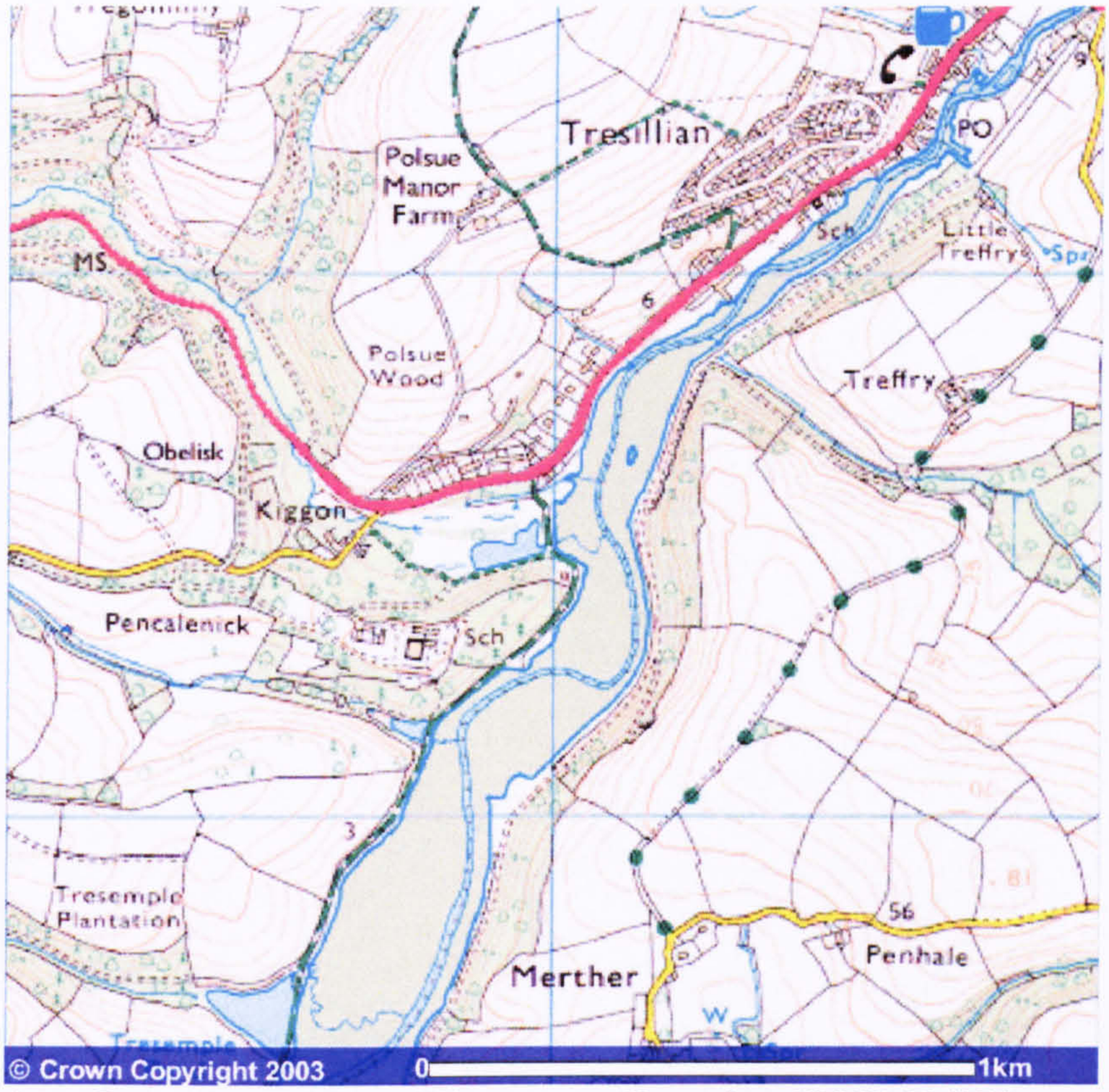


A.vi: Location of Hayle Estuary sampling site (SW555375)

A.vii: Location of Restronguet Creek sampling site (SW804385)

A.viii: Location of St. Michael's Mount sampling site (SW514299)

A.ix: Location of Tintagel Head sampling site (SW053893)



A.x: Location of Tresillian sampling site (SW860455)

Axi: Location of Trevone (Padstow) sampling site (SW893764)

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TECHNOLOGY DEVELOPMENT

CB/27 March 2000/BBD501/00-132

MINERALOGICAL CHARACTERISATION OF A SAMPLE OF LIHIR ORE FOR THE RIO TINTO EXTERNAL RESEARCH PROGRAMME

1 INTRODUCTION

Dr. Chris Cross from the Rio Tinto External Research Programme submitted a single sample of ore from the Lihir mine; Papua New Guinea, for mineralogical characterisation. The aim of this investigation was to document the mineralogy of the ore to provide a reference for subsequent bio-oxidation testwork to be performed at Herriot-Watt University. This note serves to summarise the results of this investigation.

2 METHODS OF INVESTIGATION

2.1 Sample Preparation

A single, representative sub-sample of ore was washed and wet screened into >500, <500>250, <250>106, <106>53 and <53µm size fractions. Each of the size fractions was subjected to a heavy liquid separation stage using a Na-polytungstate solution with a density of 2.89. The resultant heavy mineral concentrates were mounted in epoxy resin and polished in preparation for study.

2.2 Reflected Light Microscopy

Each of the polished sections was systematically examined using conventional reflected light microscopy techniques. The individual opaque phases were identified on the basis of their optical properties.

2.3 Scanning Electron Microscopy

A selected number of the polished sections were systematically examined using qualitative scanning electron microscopy techniques. This provided additional information on the compositions of individual phases and served to confirm their identities. A number of false colour, computer enhanced backscattered electron images were also prepared to illustrate important mineralogical features of the ore.

Rio Tinto Technology Development Limited
Unit 34, Hither Green Industrial Estate, Clevedon, North Somerset. BS21 6XU
Telephone +44 1275 874344 *Fax* +44 1275 874337
REGISTERED OFFICE: 6 St James's Square London SW1Y 4LD Registered in England No. 221805

2.4 Grain Size Distribution

The grain size distribution of the ore sample, as determined by wet screening, together with the results of the heavy liquid separation is summarised in Table 1.

Table 1 - Grain Size Distribution and Heavy Mineral Content

Size Fraction (µm)	Distribution (Wt. %)	D>2.89 (Wt. %)
>500	9.2	3.8
<500>250	18.2	5.8
<250>106	14.1	15.8
<106>53	11.5	24.1
<53	47.0	-
Total	100.0	

The results of the wet screening confirm that a significant proportion of the ore (47.0%) reports to the <53µm size fraction. The heavy mineral content of the ore is variable, with a maximum of 24.1% of the ore in the <106>53µm size fraction reporting to the heavy mineral concentrate. The <53µm size fraction was not subjected to heavy liquid separation.

3 CHEMISTRY

A sample of the ore was submitted to the Bondar-Clegg Laboratory for assay and the results are summarised in Table 2. The ore is characterised by a high silica content (56.75%) that reflects the presence of significant amounts of K-feldspar and quartz [SiO_2] in addition to subordinate amounts of fine-grained muscovite (more commonly referred to as *sericite*) [ideally $KAl_2(Si_3Al)O_{10}(OH)_2$], chlorite [probably chamosite ideally $(Fe^{2+},Mg,Fe^{3+})_5Al(Si_3Al)O_{10}(OH,O)_8$] and clay minerals that are dominated by kaolinite [ideally $Al_2Si_2O_5(OH)_4$].

K-feldspar, together with the aluminosilicate minerals also account for the great bulk of the alumina content (16.02%) as well as the alkalis K_2O (7.38%) and Na_2O (0.40%). Subordinate amounts of plagioclase [$NaAlSi_3O_8$– $CaAl_2Si_2O_8$] are also present. The small amount of TiO_2 (0.73%) reflects the presence of accessory amounts of rutile [TiO_2]. The MgO content (0.98%) is moderate and largely reflects the presence of small amounts of chlorite and a variety of Mg-bearing carbonate minerals including ferroan dolomite [$CaMg(CO_3)_2$], siderite [$Fe(CO_3)$] and rhodochrosite [$MnCO_3$]. The bulk of the CaO (0.99%) is also present in the form of carbonate minerals.

Table 2 – Chemistry

Oxide/Element	(%)	Element	(ppm)
SiO_2	56.75	Au	6.35
TiO_2	0.73	Ag	1.4
Al_2O_3	16.02	As	1617
MgO	0.98	Sb	12
CaO	0.99	Ba	374
K_2O	7.38	Co	37
Na_2O	0.40	Cr	121
Cu	0.03	Hg	0.5
Fe (total)	7.76	Mn	1208
S	7.26	Ni	84
		P	2091
		Pb	87
		Zn	254

The Cu content (0.03%) is low and reflects the presence of very minor amounts of chalcopyrite [$CuFeS_2$]. The bulk of the Fe (7.76%) is present in the form of pyrite [FeS_2] with subordinate amounts being contributed by arsenopyrite [FeAsS] and the various Fe-bearing varieties of chlorite. The great bulk of the sulphur (7.26%) in the ore is present as pyrite, with subordinate amounts also being present in arsenopyrite.

The ore is characterised by significant amounts of Au (6.35ppm) with lesser Ag (1.4ppm) most probably present largely in solid solution within the pyrite and arsenopyrite. The As content (1617ppm) is significant and reflects the presence of As-zoned pyrite and subordinate arsenopyrite. The Sb content (12ppm) is low and may reflect the presence of trace amounts of tetrahedrite [$Cu_{12}Sb_4S_{13}$]. Sb may also occur in solid solution within arsenopyrite. Ba (374ppm) is present as barytes [$BaSO_4$] that may also contain significant amounts of strontium. The traces of Co (37ppm) are presumably present in solid solution within pyrite with Pb (87ppm) and Zn (254ppm) reflecting the presence of traces of galena [PbS] and sphalerite [ideally ZnS] respectively.

The Cr (121ppm) and Ni (84ppm) contents, to a large extent, reflect contamination from the crushing and milling equipment used during sample preparation. Manganese (1208ppm) is present in the variety of carbonate minerals observed during this investigation. The P (2091ppm) content is moderately high and occurs largely in the form of accessory apatite [$Ca_5(PO_4)_3(F,Cl,OH)$]. The minor amount of Hg (0.5ppm) is presumably hosted by traces of sphalerite and pyrite.

4 MINERALOGY

4.1 Introduction

The mineralogical characterisation of this sample was based on the examination of heavy mineral concentrates. Heavy liquid separation of the size fractions provided a significant degree of concentration of the high-density ore minerals. The bulk of this sample, however, consists of low-density transparent gangue minerals that occur within the heavy mineral concentrates in relatively minor amounts. Therefore, the <53µm size fraction was analysed using x-ray powder diffraction techniques to confirm the identity of the dominant transparent gangue minerals.

Previous investigations have found that the great bulk of the gold within the Lihir ore is present as a highly refractory form, occurring as lattice-bound gold associated with arsenic-rich zones in pyrite. The mine currently operates a whole-ore pressure leach system to decompose the sulphide prior to cyanidation. Subordinate amounts of gold may also occur as lattice-bound gold in arsenopyrite. The lattice bound gold is typically present at levels that are below the detection limits for EDX (~0.5%) and WDX (~300ppm) techniques currently utilised on the Clevedon SEM.

4.2 Transparent Gangue Mineralogy

X-ray powder diffraction analysis of a small, representative portion of the <53µm size fraction confirms that the transparent gangue mineralogy of this ore consists predominantly of orthoclase (ideally $KAlSi_3O_8$), kaolinite (ideally $Al_2Si_2O_5(OH)_4$) and pyrite. Examination of the heavy mineral concentrates also confirmed the presence of subordinate amounts of quartz, clinopyroxene (probably augite, ideally $(Ca,Mg,Fe)_2Si_2O_6$), chlorite, Sr-bearing barytes (ideally $BaSO_4$), apatite, rutile and sericite (fine-grained muscovite) that occur largely as incompletely liberated grains that remain partially intergrown with the high-density sulphide minerals (Figure 1). In addition, a variety of carbonate minerals were also recognised. These include various Mn, Fe, Mg and Ca-rich varieties of dolomite, siderite and rhodochrosite.

4.3 Ore Mineralogy

4.3.1 Introduction

Detailed examination of the heavy mineral concentrates confirm that they consist predominantly of complexly zoned As-bearing pyrite together with subordinate amounts of arsenopyrite and incompletely liberated transparent gangue minerals.

4.3.2 Pyrite

Pyrite is the dominant sulphide mineral and occurs as euhedral crystals and as granular, fine-grained, porous or botryoidal aggregates (Figures 1 to 4). A small proportion of the pyrite also occurs as lath-like pseudomorphs after marcasite (Figure 2a). The lath-like marcasite pseudomorphs are typically porous in nature and may be overgrown by later

generations of botryoidal or euhedral pyrite that typically exhibits some degree of compositional zoning. The complex nature of the pyrite indicates a variable depositional history highlighted by significant variations in the arsenic content of the discrete pyrite layers (Figure 2b). Fine-grained arsenopyrite crystals may also be intimately intergrown with the pyrite and typically develop along discrete As-rich zones in the pyrite aggregates (Figure 3b).

X-ray dot maps were also prepared to illustrate the variation in chemistry in the complexly zoned pyrite grains (Figure 4). The x-ray dot maps illustrate the variation in Fe, As and S content in a pyrite and arsenopyrite aggregate and confirm that the compositional zoning observed in the pyrite reflect substitution of sulphur by arsenic. Pyrite is a known host for lattice-bound gold, a highly refractory, sub-micrometre occurrence of gold that is often concentrated within the arsenic-rich zones in the pyrite. Previous investigations of the Lihir gold ore have confirmed the presence of trace amounts of gold within discrete As-rich zones in the pyrite using the wavelength dispersive techniques. This techniques on the SEM has a lower limit of detection of ~300ppm. Similar studies using this technique on the current ore, however, failed to reveal the presence of detectable levels of gold within the pyrite. It is, nonetheless, still assumed that the bulk of the gold in this ore is present as lattice-bound gold within pyrite and, to a lesser extent, arsenopyrite.

4.3.3 Arsenopyrite

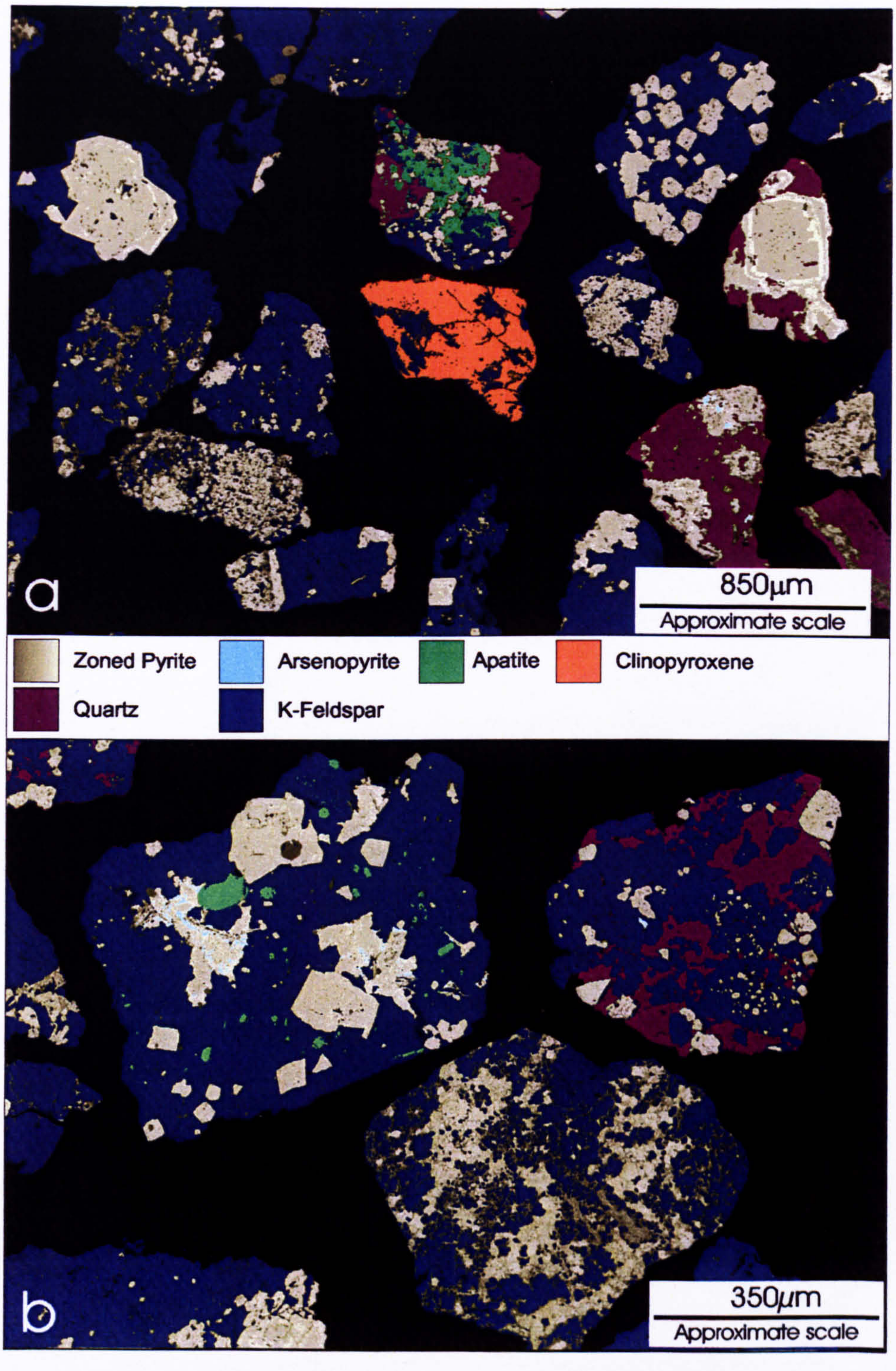
Arsenopyrite is a common accessory mineral and typically occurs as fine-grained, euhedral crystals that rarely exceed 25µm in size. The bulk of the arsenopyrite is extremely fine-grained in nature and typically occurs along arsenic-rich zones in pyrite. The arsenopyrite may also exhibit some degree of compositional zoning within discrete crystals (Figure 4a).

4.3.4 Accessory Phases

A number of accessory phases were observed in this sample in minor amounts. These include chalcopyrite and sphalerite, which typically occur as inclusions within the pyrite aggregates. Minor amounts of galena were also recognised, and may exhibit some degree of oxidation and replacement by anglesite (ideally $PbSO_4$).

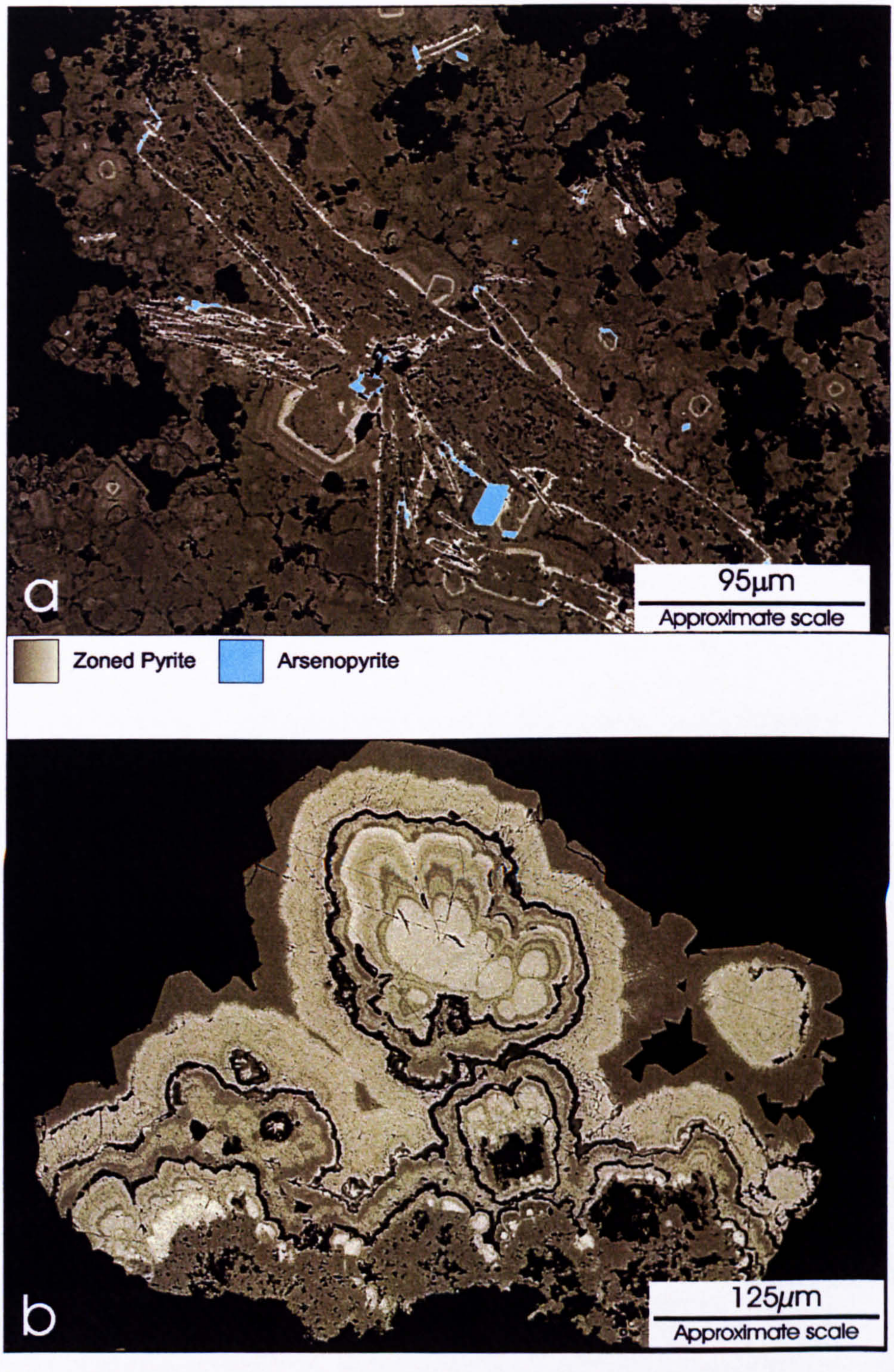
Chris Blake
MINERALOGIST
RIO TINTO TECHNOLOGY DEVELOPMENT

Figure 1



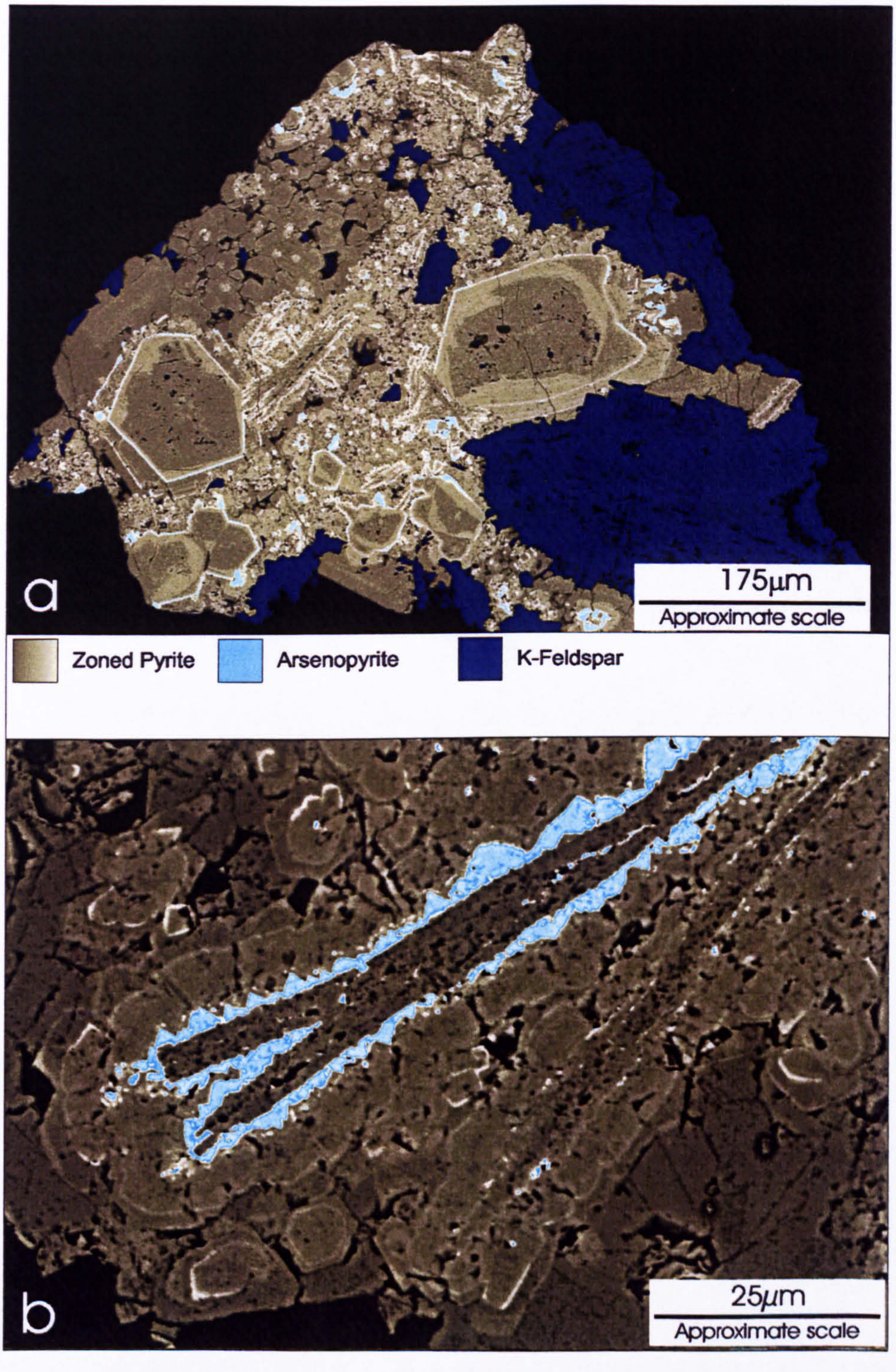
False colour, backscattered electron images illustrating (**a**) and (**b**) the general nature and appearance of the intergrowths between pyrite (light khaki-grey) and K-feldspar (dark blue). K-feldspar is the dominant transparent gangue mineral in the heavy mineral concentrates. Minor amounts of arsenopyrite (cyan) are intergrown with the pyrite. Accessory minerals include clinopyroxene (orange/brown) and apatite (green). Quartz (mauve) is also present in significant amounts.

Figure 2



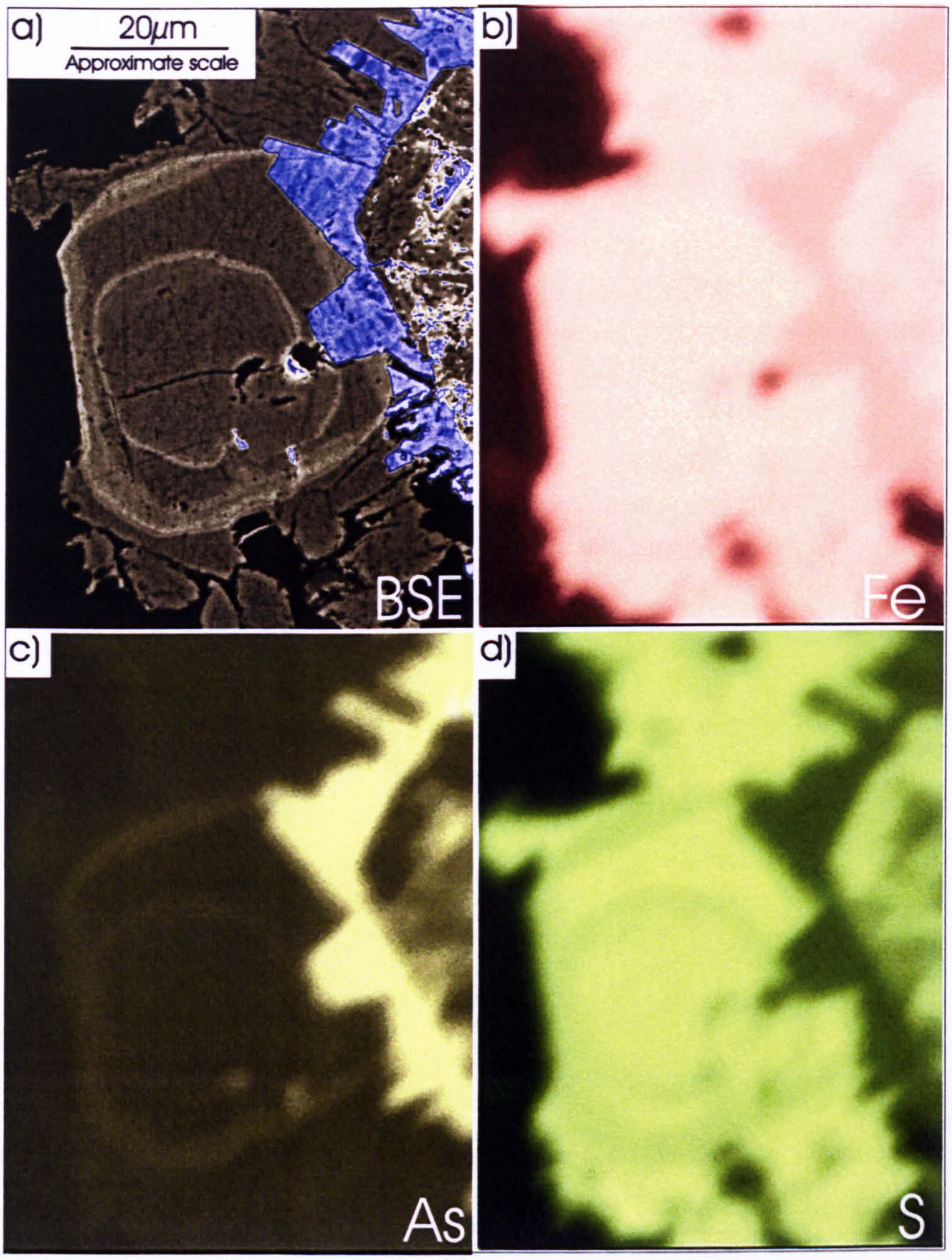
False colour, backscattered electron images illustrating (**a**) and (**b**) the extremely complex nature of the As-rich zones (lightest khaki-grey shades) in pyrite/marcasite aggregates (khaki-grey shades). Porous marcasite lamellae (a) are typically overgrown by later stages of zoned pyrite. The marcasite is largely free from As-zoning. The brighter areas represent higher concentrations of arsenic. Lattice-bound gold is also typically more abundant within these zones, but was not detected during this study.

Figure 3



False colour, backscattered electron images illustrating (**a**) the complex nature of the As-rich zones (lightest khaki-grey shades) in euhedral pyrite (khaki-grey shades). The pyrite remains intergrown with K-feldspar (dark blue). Arsenopyrite is present within the more As-rich zones in the pyrite. (**b**) Tiny arsenopyrite crystals (cyan) that are developed within As-rich pyrite, surrounding a porous marcasite lamellae (darker khaki-grey).

Figure 4



a) A false colour backscattered electron image illustrating complex compositional zoning within pyrite (khaki grey shades) and arsenopyrite (cyan). False colour x-ray dot maps illustrating the relative abundance of **b)** iron **c)** arsenic and **d)** sulphur as determined by EDX analysis. Brighter areas represent higher concentrations of the determined element. WDX analysis of this grain failed to reveal the presence of detectable amounts of Au within the As-rich pyrite.

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CB/5 March 2000/BBD501/00-091

MINERALOGICAL CHARACTERISATION OF A SAMPLE OF ESCONDIDA SULPHIDE ORE FOR RIO TINTO EXTERNAL RESEARCH PROGRAMME

1 INTRODUCTION

Dr. Chris Cross from the Rio Tinto External Research Programme submitted a single sample of ore from the Escondida Mine, Chile, for mineralogical characterisation. The aim of this investigation was to document the mineralogy of the ore to provide a reference for subsequent bio-oxidation testwork to be performed at Herriot-Watt University. This note serves to summarise the results of this investigation.

2 METHODS OF INVESTIGATION

2.1 Sample Preparation

A single, representative sub-sample of ore was washed and wet screened into >150, <150>106, <106>53, <53>25 and <25µm size fractions. Each of the size fractions was subjected to a heavy liquid separation stage using a Na-polytungstate solution with a density of 2.89. The resultant heavy mineral concentrates were mounted in epoxy resin and polished in preparation for study.

2.2 Reflected Light Microscopy

Each of the polished sections was systematically examined using conventional reflected light microscopy techniques. The individual opaque phases were identified on the basis of their optical properties. A number of colour, reflected light photomicrographs were also captured using a Buehler Omnimet 'Enterprise' image analysis system to illustrate some of the more important features of the ore.

Rio Tinto Technology Development Limited
Unit 34, Hither Green Industrial Estate, Clevedon, North Somerset. BS21 6XU
Telephone +44 1275 874344 *Fax* +44 1275 874337
REGISTERED OFFICE: 6 St James's Square London SW1Y 4LD Registered in England No. 221805

2.3 Scanning Electron Microscopy

A selected number of the polished sections were systematically examined using qualitative scanning electron microscopy techniques. This provided additional information on the compositions of individual phases and served to confirm their identities. A number of false colour, computer enhanced backscattered electron images were also prepared to illustrate important mineralogical features of the ore.

2.4 Grain Size Distribution

The result of the grain size distribution of the ore sample, as determined by wet screening, together with the results of the heavy liquid separation is summarised in Table 1.

Table 1 - Grains Size Distribution and Heavy Mineral Content

Size Fraction (µm)	Distribution (Wt. %)	D>2.89 (Wt. %)
>150	4.4	0.2
<150>106	18.5	1.2
<106>53	22.5	1.7
<53>25	13.8	1.1
<25	40.8	1.1
Total	100.0	5.3

The results of the wet screening confirm that a significant proportion of the ore (40.8%) reports to the <25µm size fraction. The heavy mineral content of the ore is also particularly low, with a total of only 5.3% of the ore reporting to the heavy mineral concentrates. This is typical of a porphyry copper ore that typically consists predominantly of low-density gangue (largely quartz and the alteration products of feldspar minerals) together with relatively minor amounts of ore minerals (largely pyrite and Cu-bearing sulphides).

3 CHEMISTRY

A sample of the ore was submitted to the Bondar-Clegg Laboratory for assay and the results are summarised in Table 2. The ore is characterised by a very high silica content (78.14%) that reflects the presence of significant amounts of quartz [SiO_2] in addition to a variety of silicate minerals, notably a fine grained variety of muscovite that is referred to as *sericite* [$KAl_2(Si_3Al)O_{10}(OH)_2$], and clay minerals that are dominated by kaolinite [ideally $Al_2Si_2O_5(OH)_4$]. These aluminosilicate minerals also account for the great bulk of the alumina (13.93%) as well as the alkalis K_2O (2.83%) and Na_2O (0.65%). Small amounts of relict K-feldspar [$KAlSi_3O_8$] and sodic plagioclase [$NaAlSi_3O_8$] are also present and contribute subordinate amounts of these oxides. The small amount of TiO_2 (0.27%) reflects the presence of accessory amounts of rutile [TiO_2] with the MgO (0.38%) being present in the form of small amounts of chlorite, the composition of which

may be expressed as $(Mg,Fe)_5Al(Si_3Al)O_{10}(OH,O)_8$. The bulk of the CaO (0.11%) is present in the form of gypsum [$CaSO_4.2H_2O$].

Table 2 – Chemistry

Oxide/Element	(%)	Element	(ppm)
SiO_2	78.14	Au	0.06
TiO_2	0.27	Ag	2.5
Al_2O_3	13.93	As	68
MgO	0.38	Ba	503
CaO	0.11	Co	7
K_2O	2.83	Cr	203
Na_2O	0.65	Hg	0.041
Cu	1.91	Mn	110
Fe (total)	2.23	Ni	99
S	2.23	P	263
		Pb	87
		Zn	72

The Cu content (1.91%) is moderate for an ore of this nature and is largely present in the form of chalcocite [Cu_2S] with subordinate amounts being present in a variety of Cu-minerals, notably digenite [$Cu_{1.80}S$], covellite [CuS], bomite [Cu_5FeS_4] and relict chalcopyrite [$CuFeS_2$]. The bulk of the Fe (2.23%) is present in the form of pyrite [FeS_2] with subordinate amounts being contributed by the various Fe-bearing Cu-sulphides and chlorite. The great bulk of the sulphur (2.23%) in the ore is present as pyrite and the various Cu-sulphide minerals. Very minor amounts of accessory barytes [$BaSO_4$] and gypsum account for a minor amount of the total sulphur.

The ore is characterised by an extremely low Au content (0.06ppm) with low trace amount of Ag (92.5ppm) most probably being present largely in solid solution within the Cu-sulphide minerals and pyrite. The As content (68ppm) is also relatively low and reflects the presence of trace amounts of As-bearing sulphides, notably enargite [Cu_3AsS_4] and tennantite-tetrahedrite solid solutions [$(Cu,Fe)_{12}As_4S_{13}$–$Cu_{12}Sb_4S_{13}$]. Some Ba (503ppm) is present as barytes [$BaSO_4$] with the balance being present in solid solution within the sericite and relict feldspars. The traces of Co (7ppm) are presumably present in solid solution within the various sulphide minerals with Pb (87ppm) and Zn (72ppm) reflecting the presence of traces of galena [PbS] and sphalerite [ideally ZnS] respectively. The Cr content (203ppm) appears high, as is that of Ni (99ppm). Both of these elements are present in the crushing and milling equipment used during sample preparation and their presence may, to a large extent, reflect contamination from these sources. Manganese (110ppm) is present in minor amounts in solid solution in many Fe-bearing minerals. The P (263ppm) is present largely in the form of accessory apatite [$Ca_5(PO_4)_3(F,Cl,OH)$]. The extremely minor amount of Hg (0.041ppm) is presumably hosted by traces of sphalerite and tennantite-tetrahedrite solid solutions.

4 MINERALOGY

4.1 Introduction

This ore sample represents a supergene enriched porphyry copper deposit that consists predominantly of transparent gangue minerals, together with subordinate, but nevertheless significant amounts of pyrite and secondary copper minerals. The primary Cu mineralogy would, prior to supergene enrichment, have consisted largely of chalcopyrite (ideally $CuFeS_2$) possibly together with subordinate amounts of bornite (ideally Cu_5FeS_4). On exposure to near-surface weathering conditions, the pyrite oxidises to form Fe-oxides and hydroxides, notably hematite (ideally Fe_2O_3) and goethite (ideally α-$Fe^{3+}O(OH)$). The primary Cu-bearing sulphide minerals are also oxidised and Cu ions are released into solution. These Cu-rich, acidic solutions percolate downwards resulting in an enrichment of Cu, and other metals in a supergene zone. The supergene Cu-bearing sulphides typically replace the primary ore minerals, including chalcopyrite, sphalerite and galena. Eventually, as supergene enrichment progresses, pyrite may also be replaced.

The mineralogical characterisation of the Escondida ore sample was based on the examination of polished sections prepared from the heavy mineral concentrates. It should be noted that the heavy mineral concentrates only represent a very small proportion of the ore (~5%). This very small component of the ore, nevertheless, contains those phases that are of economic importance during mining, notably chalcopyrite and secondary Cu-sulphides.

4.2 Transparent Gangue Mineralogy

X-ray powder diffraction analysis of a small, representative portion of the <25µm Na-polytungstate float fraction confirms that the transparent gangue mineralogy of this ore consists predominantly of quartz, muscovite (ideally $KAl_2(Si_3Al)O_{10}(OH)_2$) and kaolinite (ideally $Al_2Si_2O_5(OH)_4$). Examination of the heavy mineral concentrates also confirmed the presence of quartz and sericite (fine-grained muscovite) that occur as incompletely liberated grains that remain partially intergrown with the high-density sulphide minerals. In addition, minor amounts of barytes (ideally $BaSO_4$) , rutile (ideally TiO_2) and gypsum (ideally $CaSO_4.2H_2O$) were also recognised.

4.3 Ore Mineralogy

4.3.1 Introduction

Detailed examination of the heavy mineral concentrates confirm that they consist predominantly of pyrite together with subordinate amounts of secondary Cu-bearing sulphide minerals, relict primary chalcopyrite and sphalerite.

4.3.2 Pyrite

Pyrite is the dominant sulphide mineral and occurs as anhedral crystals and angular fragments that may exceed 100µm in size (Figure 1a). The bulk of the pyrite exhibits

partial and extensive degrees of replacement by supergene, secondary Cu-sulphide minerals that typically replace the pyrite grains along fractures and grain boundaries (Figures 1a, 1b, and 2a). The pyrite grains also typically contain numerous rounded inclusions of one or more of transparent gangue and Cu-bearing sulphide minerals (Figure 2a). The Cu-sulphide inclusions within the pyrite also typically exhibit some degree of partial or extensive supergene enrichment and replacement by bornite, covellite, digenite and chalcocite.

4.3.3 Secondary Cu-Sulphides

Supergene, secondary Cu-sulphide minerals host the bulk of the Cu content of this sample. Chalcocite (ideally Cu_2S), digenite (ideally $Cu_{1.80}S$), covellite (ideally CuS) and bornite extensively replace the primary ore minerals, notably chalcopyrite and sphalerite (Figures 1b, 2b and 3a). The pyrite also exhibits partial and extensive degrees of replacement along fractures and grain boundaries by secondary Cu-sulphide minerals (Figures 1a, 1b and 2a). Chalcocite is the dominant Cu-bearing sulphide mineral and may also be intimately intergrown with other Cu-bearing sulphides (Figures 3a and 3b). Covellite is present in subordinate amounts (Figure 3a). Examination of the secondary Cu-sulphide minerals in reflected light confirms that the supergene Cu-sulphides are often complex in nature and appear to consist of intergrowths between one or more secondary sulphides (Figure 3b). These intergrowths probably consist of one or more of chalcocite, covellite, digenite and anilite (ideally Cu_7S_4). The speciation of the supergene Cu-sulphide minerals was, however, beyond the scope of the current investigation.

4.3.4 Chalcopyrite

Chalcopyrite is present in the heavy mineral concentrates in relatively minor amounts and occurs as relict primary grains that have largely been replaced by supergene Cu-sulphide minerals, notably chalcocite, covellite and digenite-like phases (Figures 1b, 3a and 3b). The chalcopyrite may also occur as small, rounded inclusions in pyrite. These inclusions have, however, also typically been subjected to some degree of supergene alteration and replacement (Figure 2b).

4.3.5 Accessory Phases

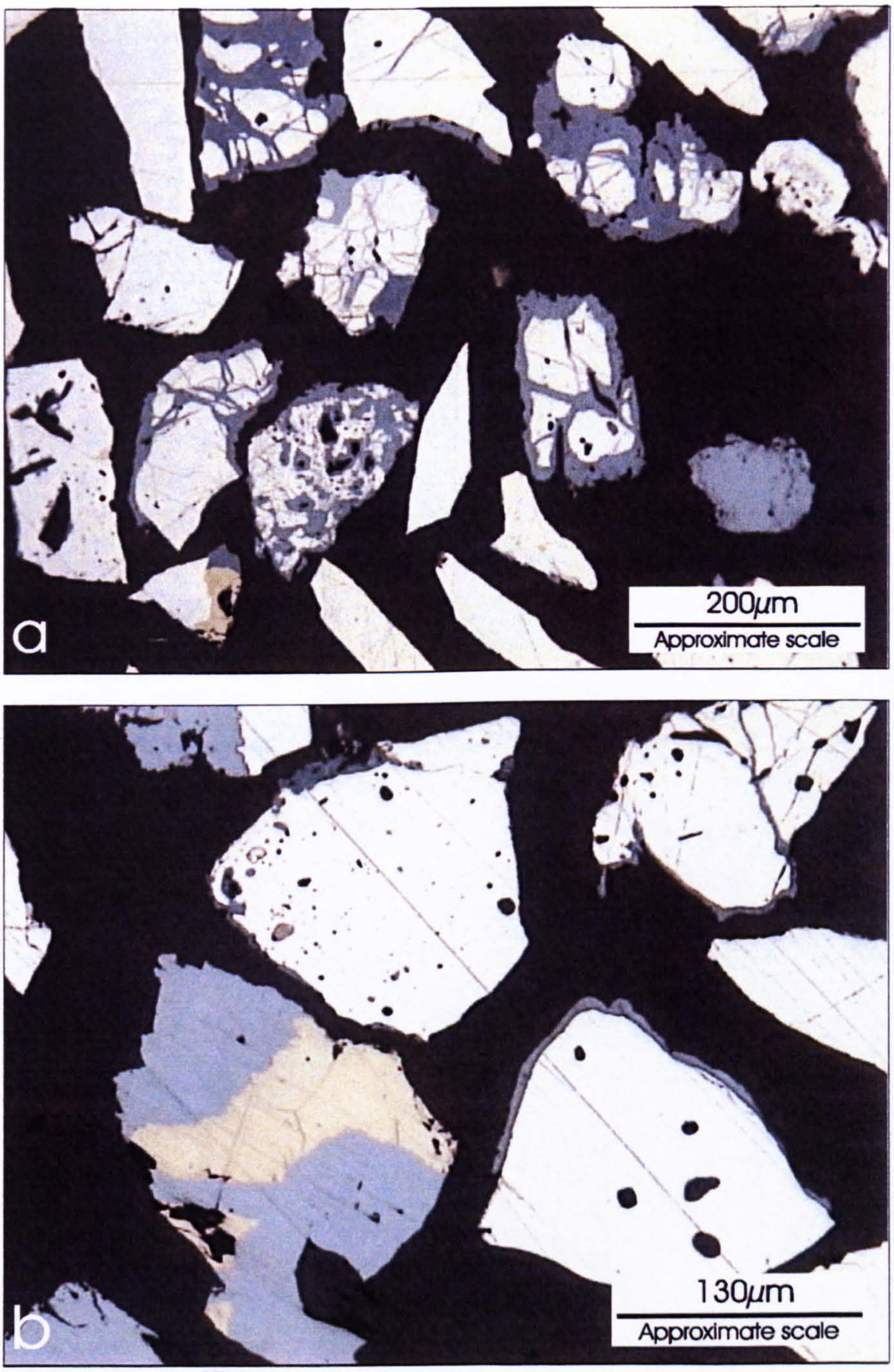
Members of the tetrahedrite-tennantite (ideally $Cu_{12}Sb_4S_{13}$–$(Cu,Fe)_{12}As_4S_{13}$) solid solution series are common accessory phases and probably represent components of the primary mineralogy. These phases have, however, largely been replaced by secondary Cu-sulphides (Figures 4a and 4b). Enargite (ideally Cu_3AsS_4) is also present in minor amounts and also appear to largely represent a component of the primary mineralogy. Again, the bulk of the primary enargite exhibits some degree of replacement by secondary Cu-sulphide minerals (Figures 5a, 5b and 6a). Enargite may also occur in supergene deposits as a secondary mineral, and may, at least in part, also form a component of the supergene mineralogy of this ore.

Molybdenite (ideally MoS_2) is relatively common and occurs as flake-like grains that may exceed 150µm in maximum dimension (Figure 6b). The bulk of the molybdenite

observed in this sample occurs as discretely liberated grains. Sphalerite is present as rare relict grains that have largely been replaced by secondary Cu-sulphide minerals (Figure 3b). Galena is also rare and typically occurs as micrometre-sized inclusions in pyrite. Discrete liberated grains of galena may also occur and rarely exceed 20µm in size.

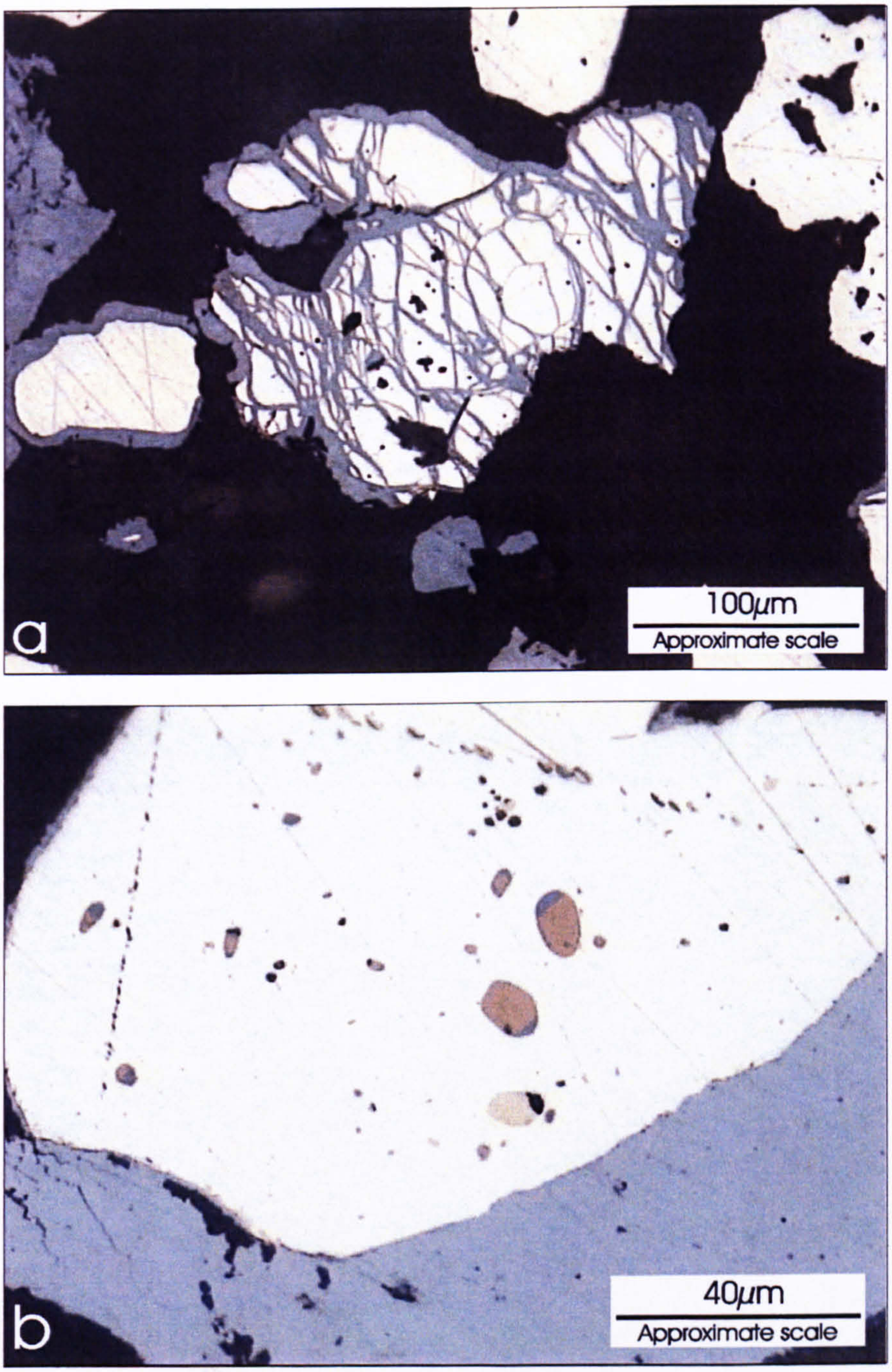
Chris Blake
MINERALOGIST
RIO TINTO TECHNOLOGY DEVELOPMENT

Figure 1



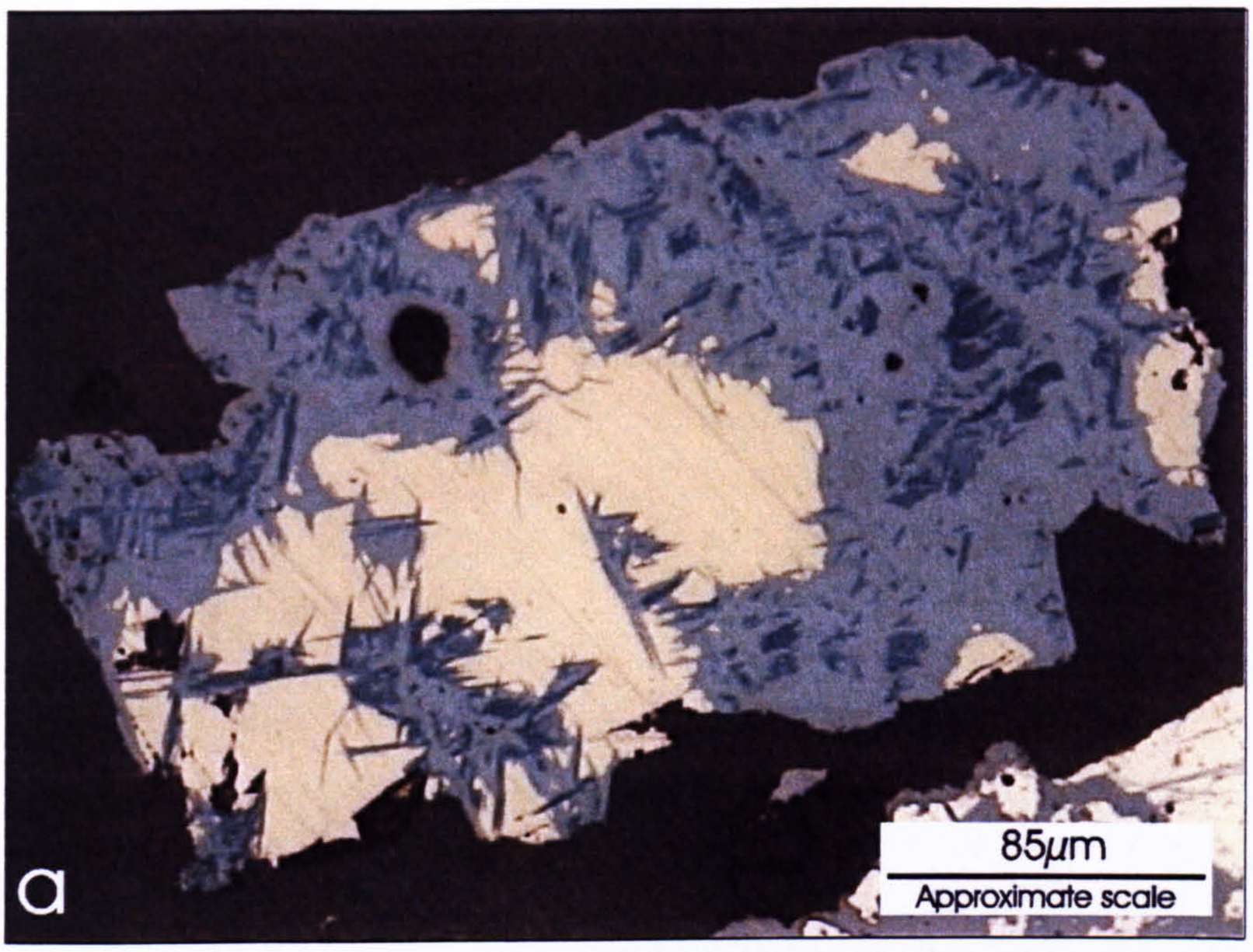
Colour reflected light photomicrographs illustrating (**a**) and (**b**) the general nature and appearance of pyrite (light cream-white) and chalcocite (medium grey). The chalcocite partially replaces the pyrite along grain boundaries and fractures. Note the presence of a chalcopyrite grain (cream-yellow) in (b) that has been extensively replaced by chalcocite.

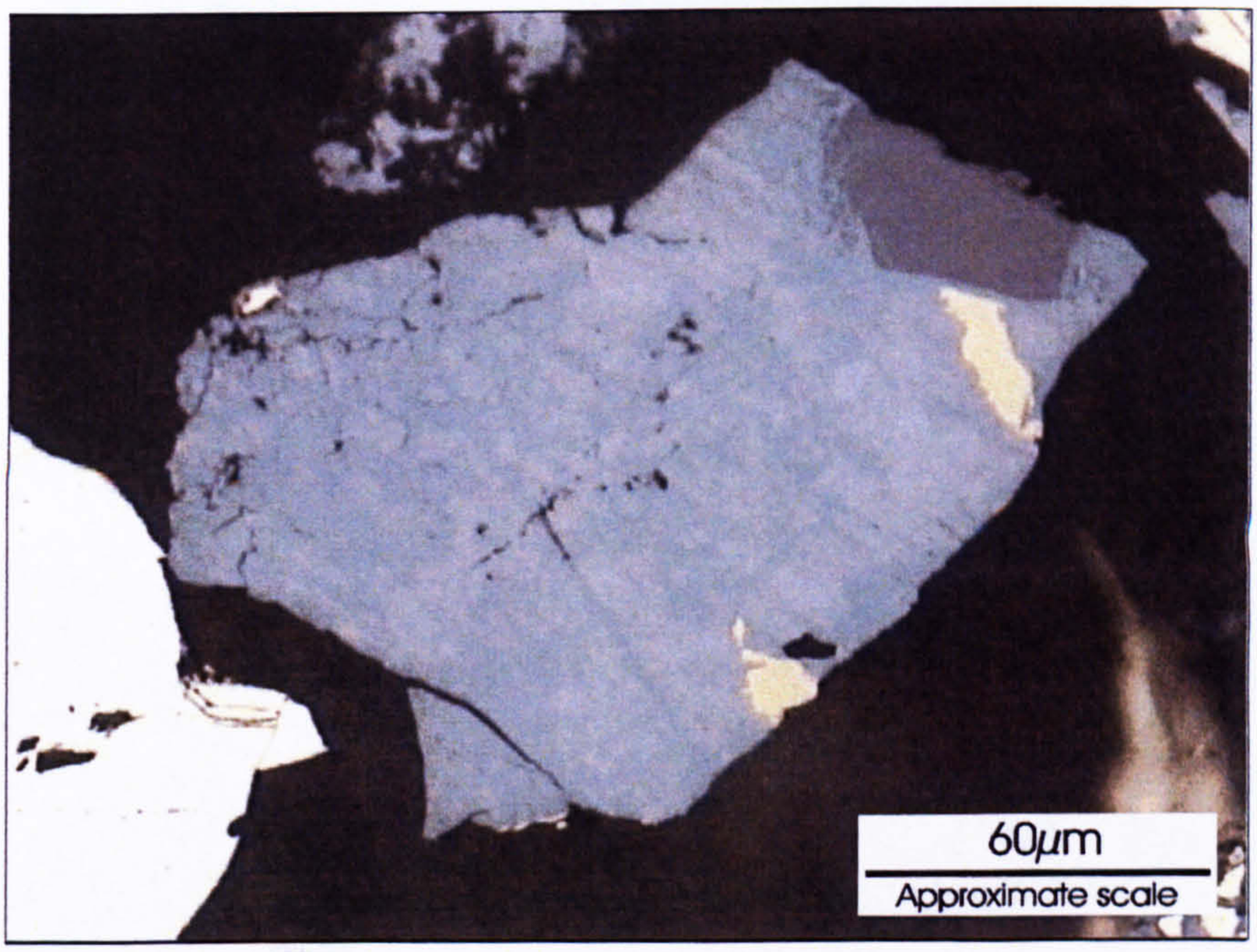
Figure 2



Colour reflected light photomicrographs illustrating (**a**) an extensively fractured pyrite grain (pale cream-white) that has been partially replaced by chalcocite (medium grey). (**b**) Small rounded inclusions of chalcopyrite (cream-yellow) and bornite (brown) in pyrite. These inclusions probably represent former chalcopyrite that has been largely replaced by bornite and chalcocite during supergene enrichment. The pyrite grain has also been partially replaced along the margins by chalcocite.

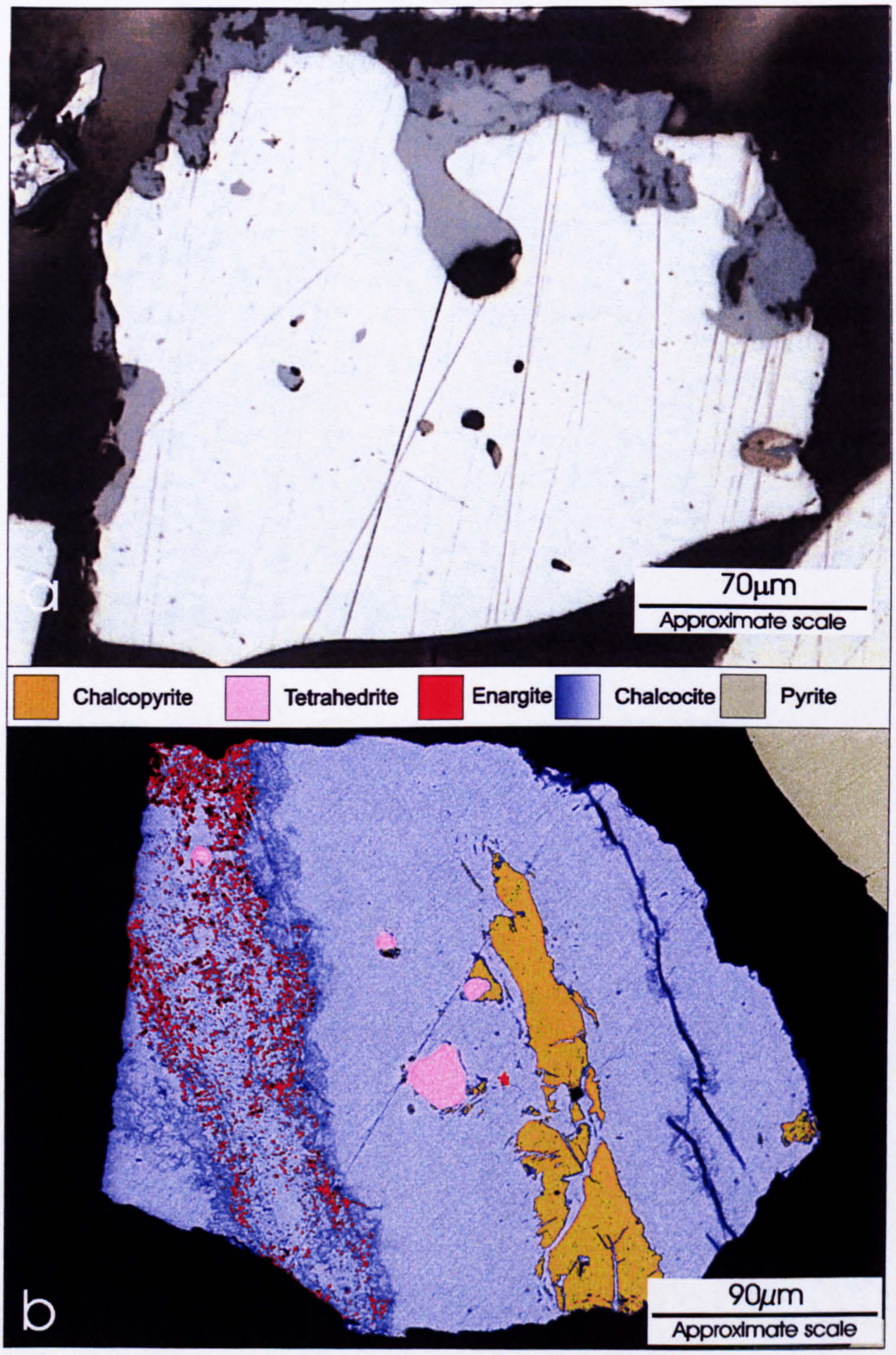
Figure 3





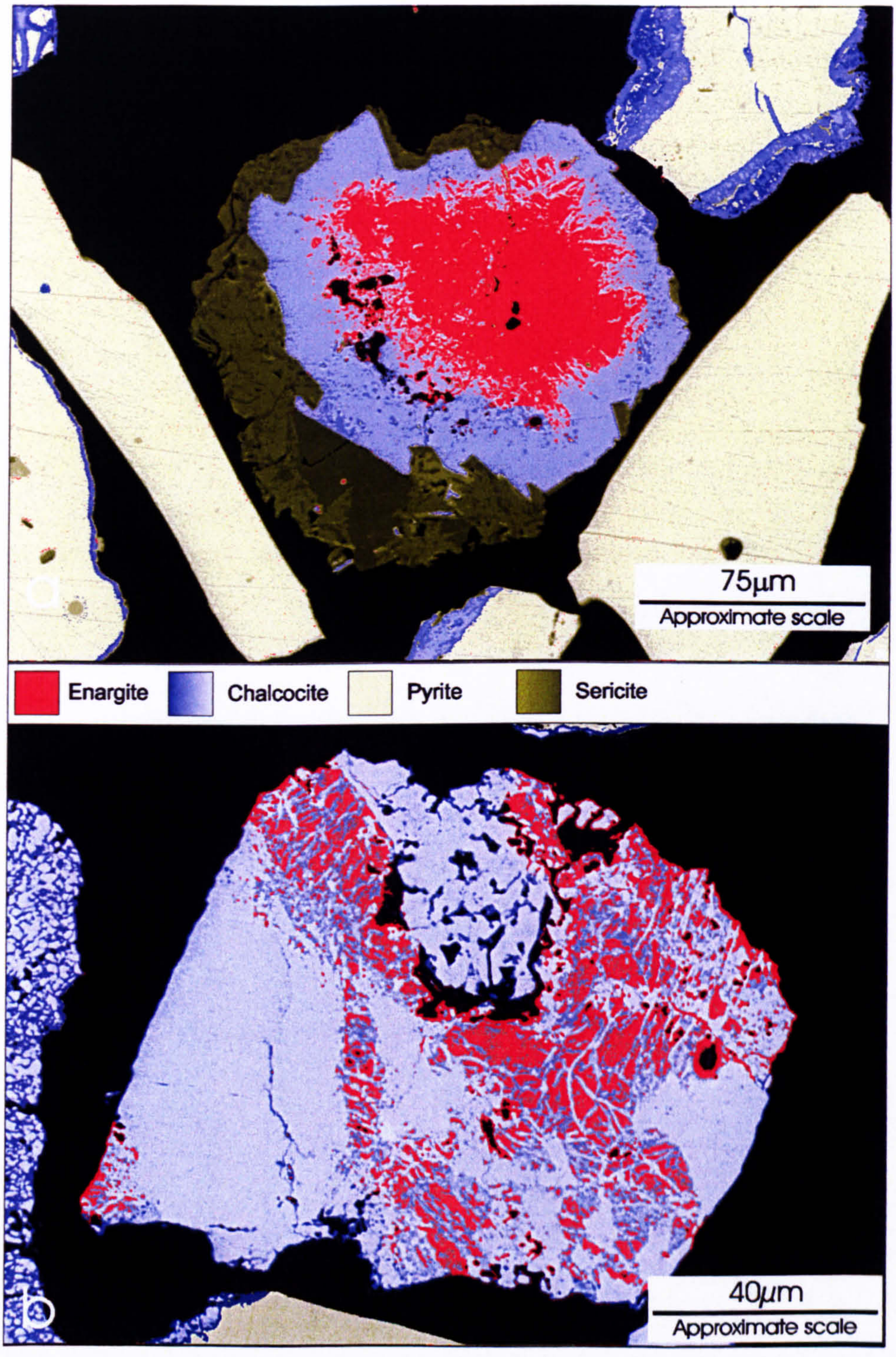
Colour reflected light photomicrographs illustrating (**a**) a chalcopyrite grain (cream-yellow) that has been extensively replaced by chalcocite (medium grey-blue) and covellite (darker grey-blue). (**b**) A sphalerite (grey) and chalcopyrite (cream-yellow) grains that has been extensively replaced by secondary Cu-sulphides (mottled grey-blue). Pyrite (pale cream-white) is also present in the field of view. The supergene Cu-sulphide probably represents integrowths between chalcocite and digenite/anilite-like phases.

Figure 4



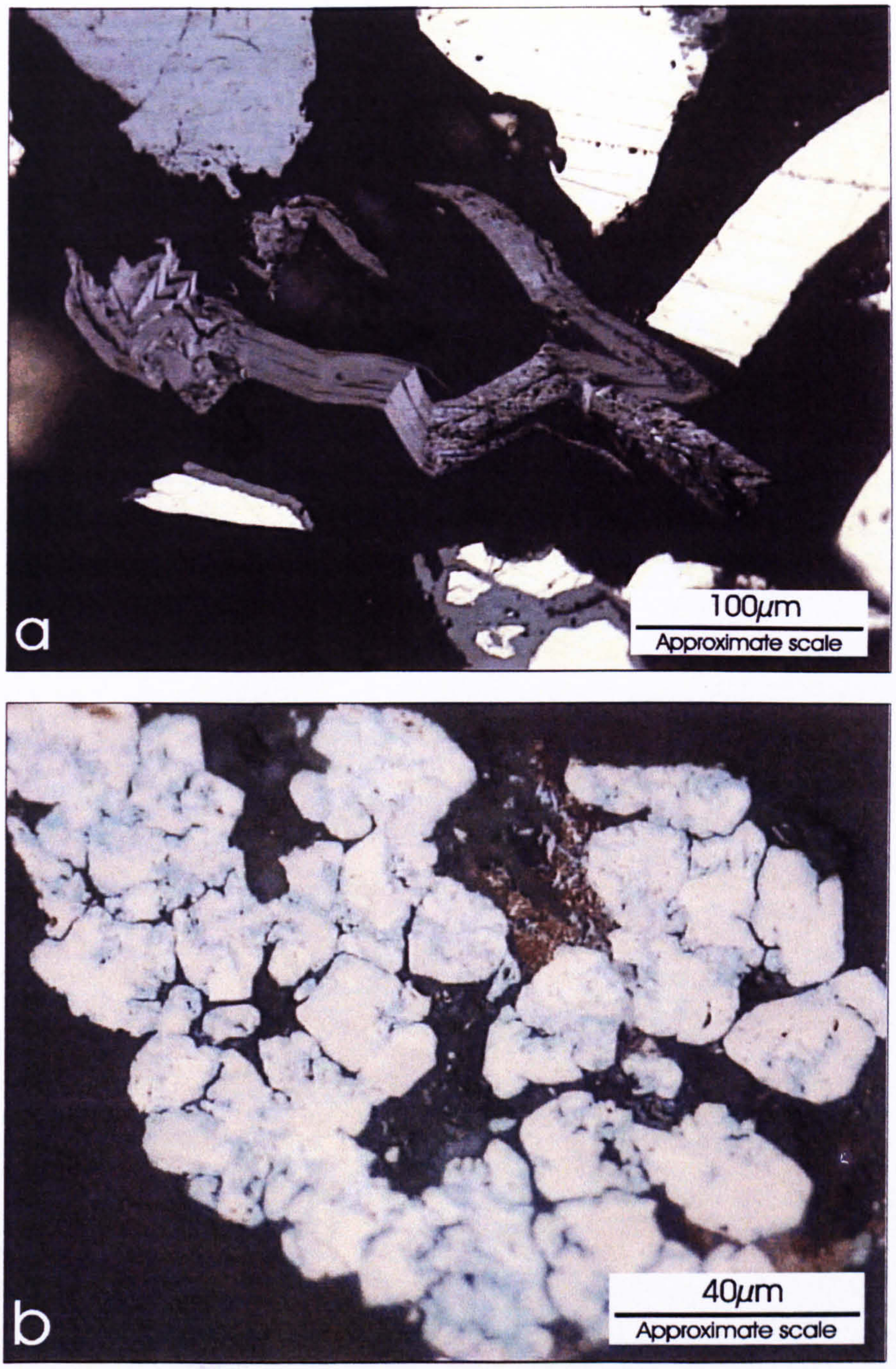
(**a**) Colour reflected light photomicrograph illustrating the partial replacement of tetrahedrite (lighter grey) and pyrite (pale cream-white) by secondary Cu-sulphides (darker grey/blue). (**b**) A false colour, backscattered electron image illustrating relict chalcopyrite (orange) and tetrahedrite (pink) that has been extensively replaced by chalcocite (blue shades). Finely intergrown enargite (red) may be relict or supergene in nature.

Figure 5



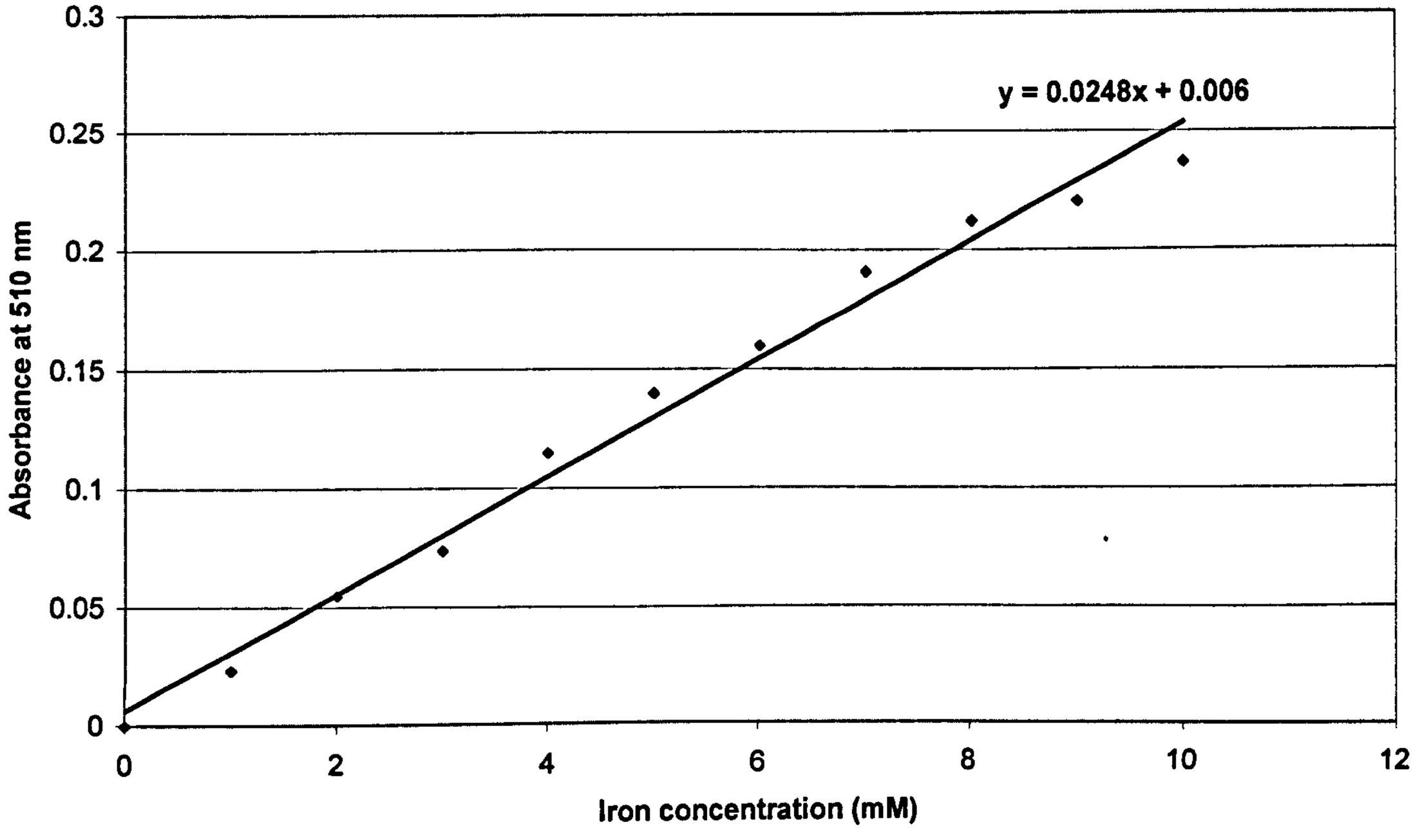
False colour, backscattered electron images illustrating (**a**) an enargite grain (red) that has been extensively replaced by chalcocite (blue). This grain remains partially intergrown with sericite (fine-grained muscovite, khaki). Pyrite (pale cream-white) also exhibits some degree of replacement by secondary Cu-sulphides. (**b**) Complex enargite intergrowths with chalcocite.

Figure 6

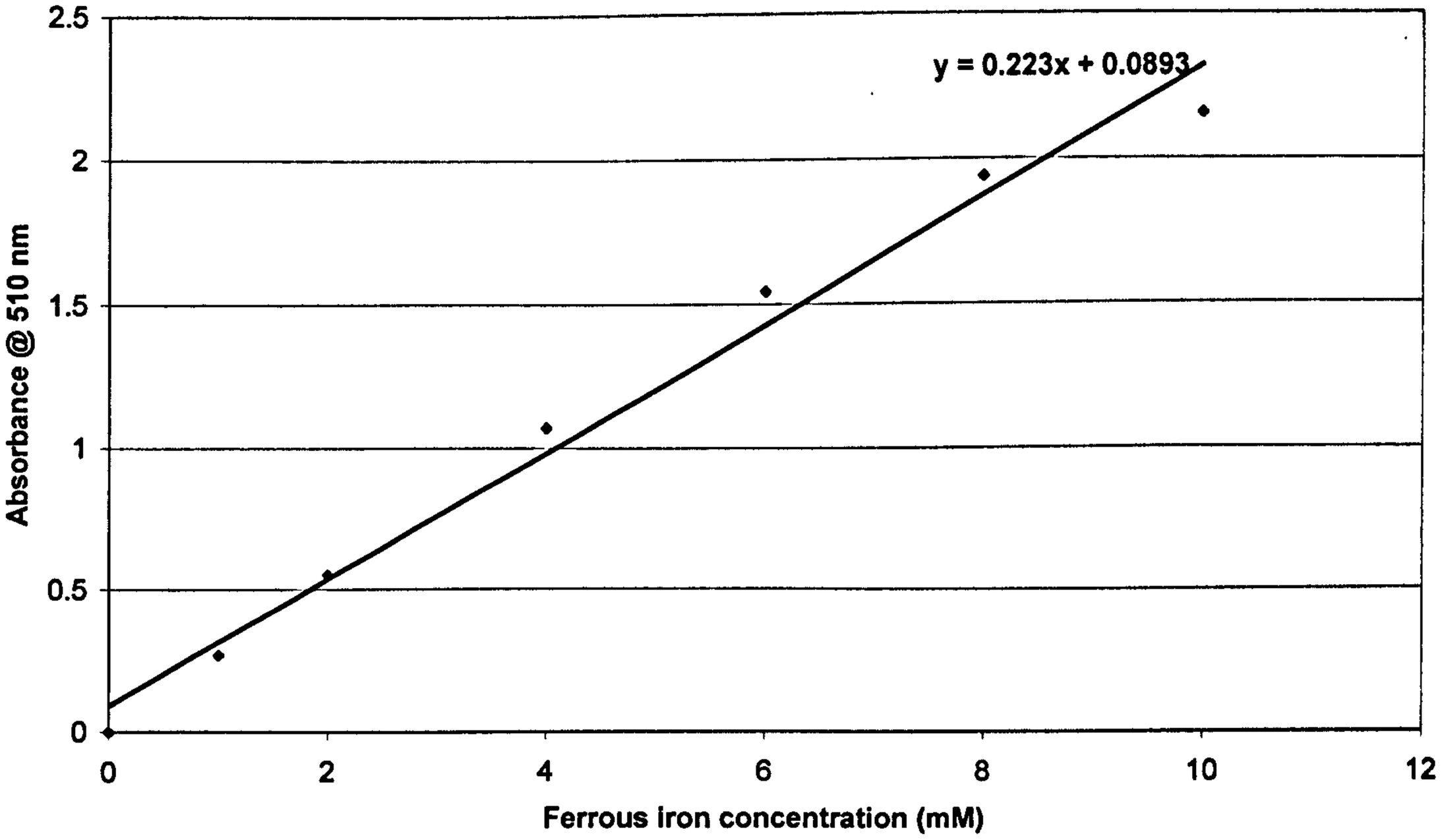


Colour reflected light photomicrographs illustrating (**a**) a liberated molybdenite particle (flake-like, grey shades). Pyrite (pale cream-white) and chalcocite (grey) are also present. (**b**) An enargite aggregate (pale grey/brown) that exhibits partial replacement by covellite (grey/blue).

Appendix C – Standard curves for the determination of ferrous and total iron concentrations



Ci – standard curve for the determination of total iron concentration using the pyrite method



Cii - Standard curve for the determination of ferrous iron concentrations using the pyrite method

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Primer oligonucleotides	MWG – BiotechAG
TSR buffer	ABI PRISM, Applied Biosystems
DNA Sequencing Kit, containing: Control DNA Control primer BigDye terminator Cycle Sequencing Ready Reaction Mix (Amplitaq DNA polymerase)	ABI PRISM, Applied Biosystems
CONCERT Rapid PCR purification system:	GIBCOBRL, LifeTechnologies
DNeasy Tissue Kit for DNA extraction http://www.qiagen.com	Qiagen